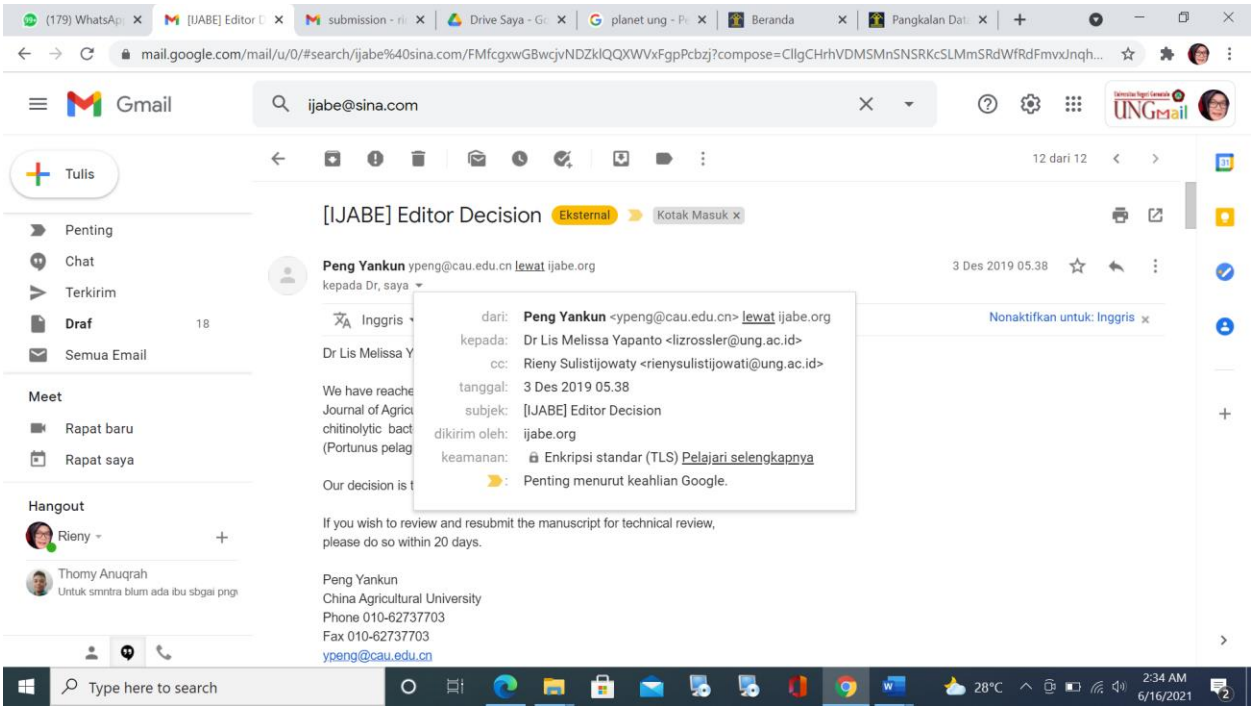

[IJABE] Editor Decision

Peng Yankun ypeng@cau.edu.cn lewat ijabe.org
kepada Dr, saya
3 Des 2019 05.38

dari: Peng Yankun <ypeng@cau.edu.cn> lewat ijabe.org
kepada: Dr Lis Melissa Yapanto <lizrossler@ung.ac.id>
cc: Rieny Sulistijowaty <rienysulistijowati@ung.ac.id>
tanggal: 3 Des 2019 05.38
subjek: [IJABE] Editor Decision
dikirim oleh: ijabe.org
keamanan: Enkripsi standar (TLS) Pelajari selengkapnya
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Dr Lis Melissa Y

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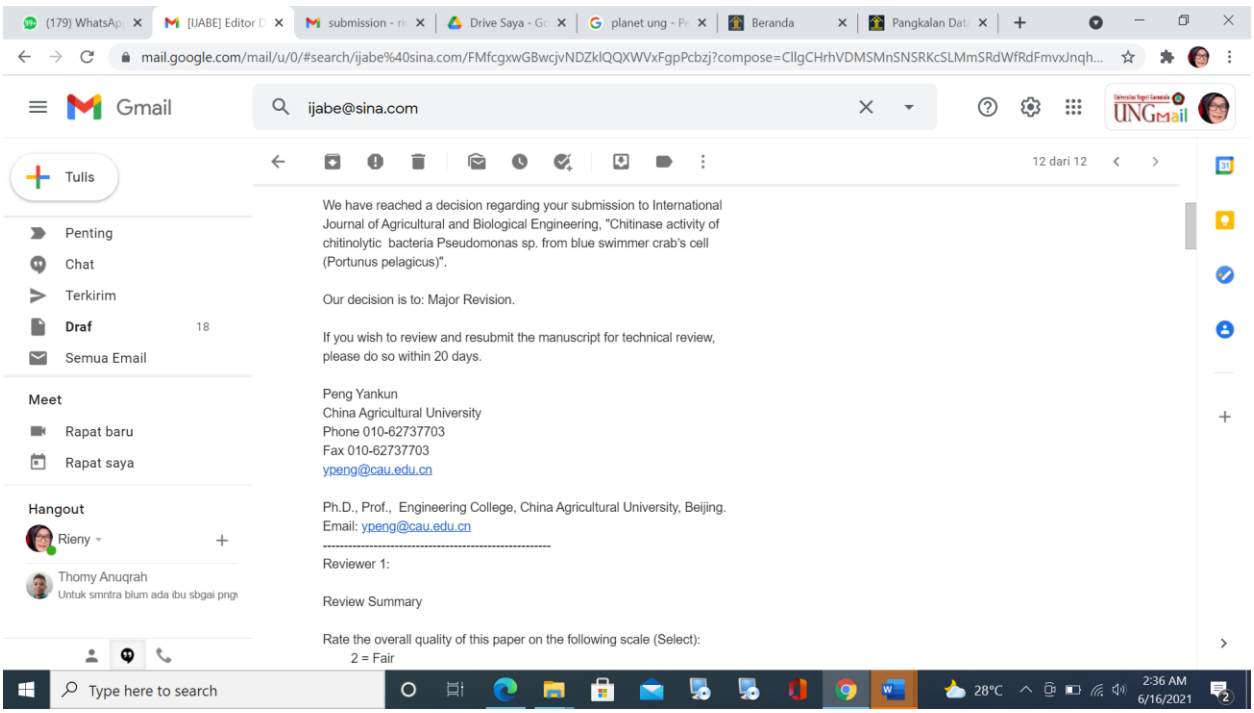
Our decision is t

If you wish to review and resubmit the manuscript for technical review, please do so within 20 days.

Peng Yankun
China Agricultural University
Phone 010-62737703
Fax 010-62737703
ypeng@cau.edu.cn



We have reached a decision regarding your submission to International Journal of Agricultural and Biological Engineering, "Chitinase activity of chitinolytic bacteria Pseudomonas sp. from blue swimmer crab's cell (Portunus pelagicus)".

Our decision is to: Major Revision.

If you wish to review and resubmit the manuscript for technical review, please do so within 20 days.

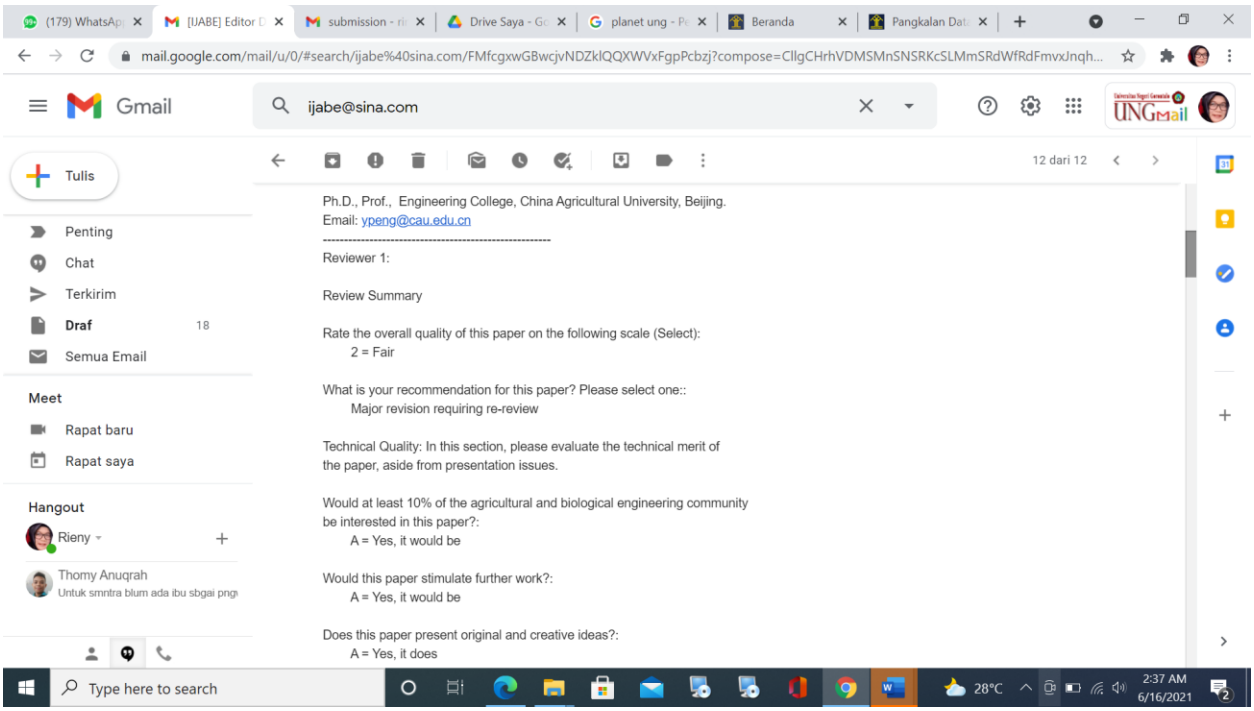
Peng Yankun
China Agricultural University
Phone 010-62737703
Fax 010-62737703
ypeng@cau.edu.cn

Ph.D., Prof., Engineering College, China Agricultural University, Beijing.
Email: ypeng@cau.edu.cn

Reviewer 1:

Review Summary

Rate the overall quality of this paper on the following scale (Select):
2 = Fair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Ph.D., Prof., Engineering College, China Agricultural University, Beijing.
Email: ypeng@cau.edu.cn
Reviewer 1:
Review Summary
Rate the overall quality of this paper on the following scale (Select):
2 = Fair
What is your recommendation for this paper? Please select one::
Major revision requiring re-review
Technical Quality: In this section, please evaluate the technical merit of the paper, aside from presentation issues.
Would at least 10% of the agricultural and biological engineering community be interested in this paper?:
A = Yes, it would be
Would this paper stimulate further work?:
A = Yes, it would be
Does this paper present original and creative ideas?:
A = Yes, it does
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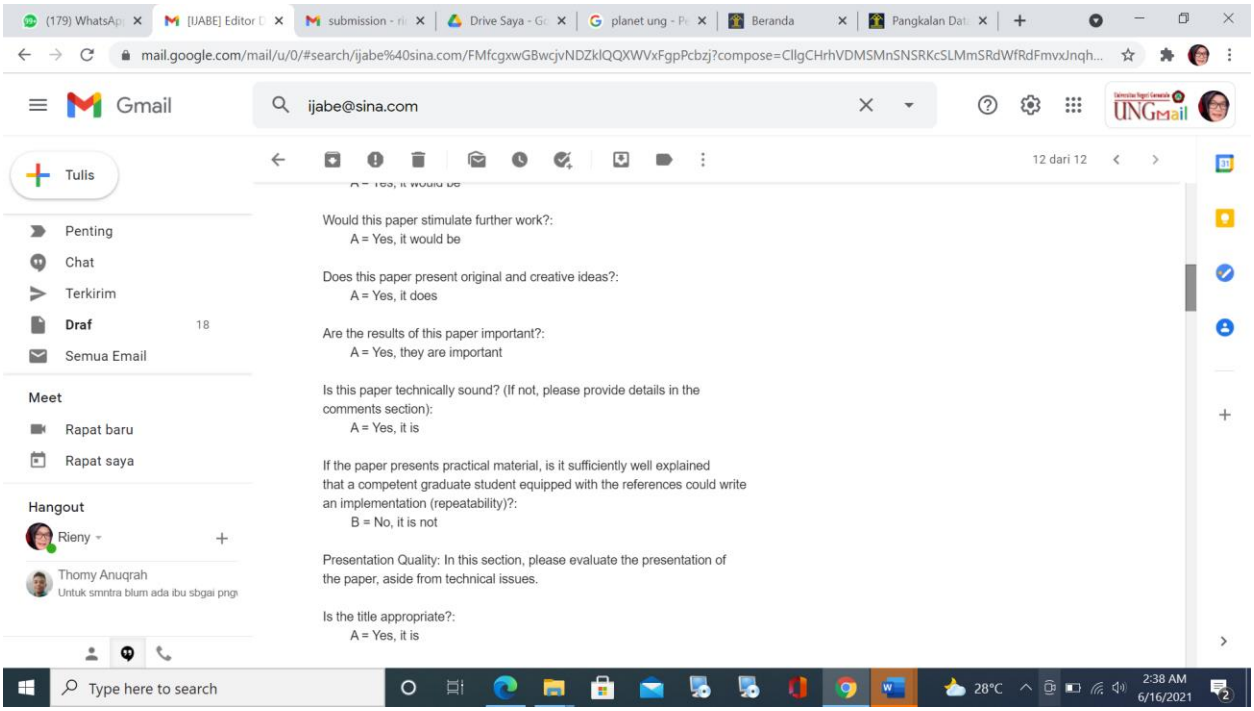
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Would this paper stimulate further work?:
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Does this paper present original and creative ideas?:
A = Yes, it does
Are the results of this paper important?:
A = Yes, they are important
Is this paper technically sound? (If not, please provide details in the comments section):
A = Yes, it is
If the paper presents practical material, is it sufficiently well explained that a competent graduate student equipped with the references could write an implementation (repeatability)?:
B = No, it is not
Presentation Quality: In this section, please evaluate the presentation of the paper, aside from technical issues.
Is the title appropriate?:
A = Yes, it is
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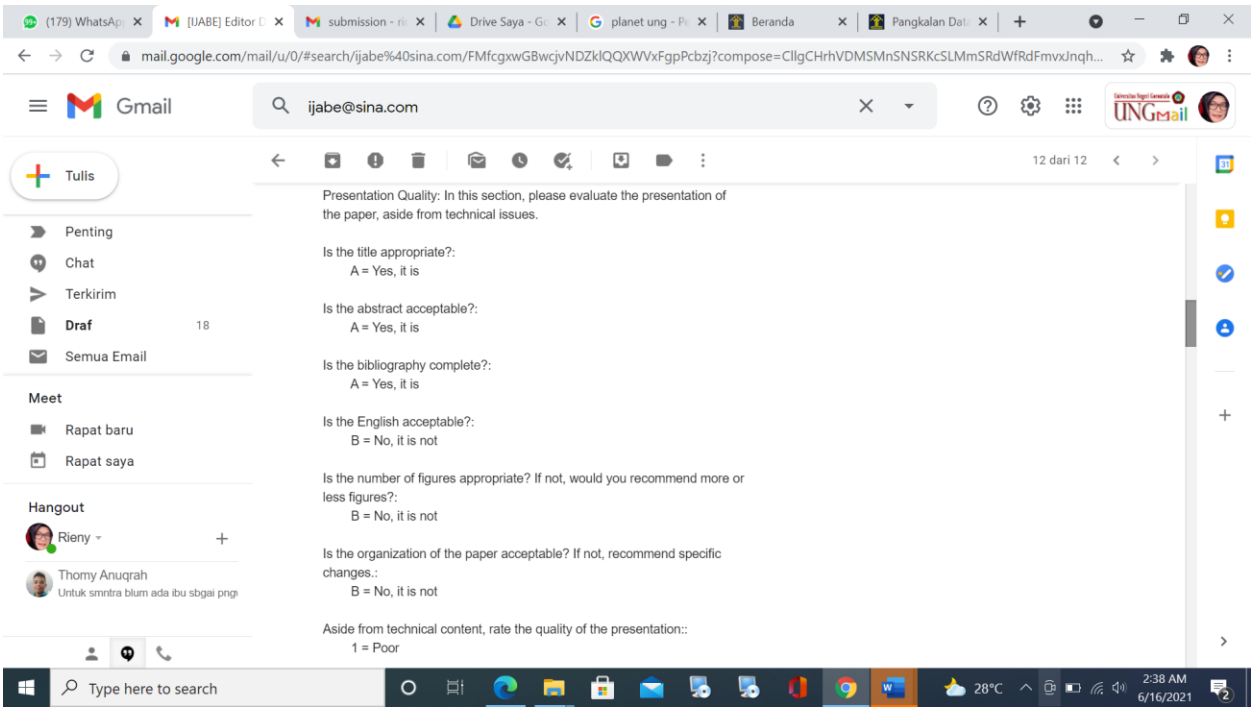
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Presentation Quality: In this section, please evaluate the presentation of the paper, aside from technical issues.
Is the title appropriate?:
A = Yes, it is
Is the abstract acceptable?:
A = Yes, it is
Is the bibliography complete?:
A = Yes, it is
Is the English acceptable?:
B = No, it is not
Is the number of figures appropriate? If not, would you recommend more or less figures?:
B = No, it is not
Is the organization of the paper acceptable? If not, recommend specific changes.:
B = No, it is not
Aside from technical content, rate the quality of the presentation::
1 = Poor
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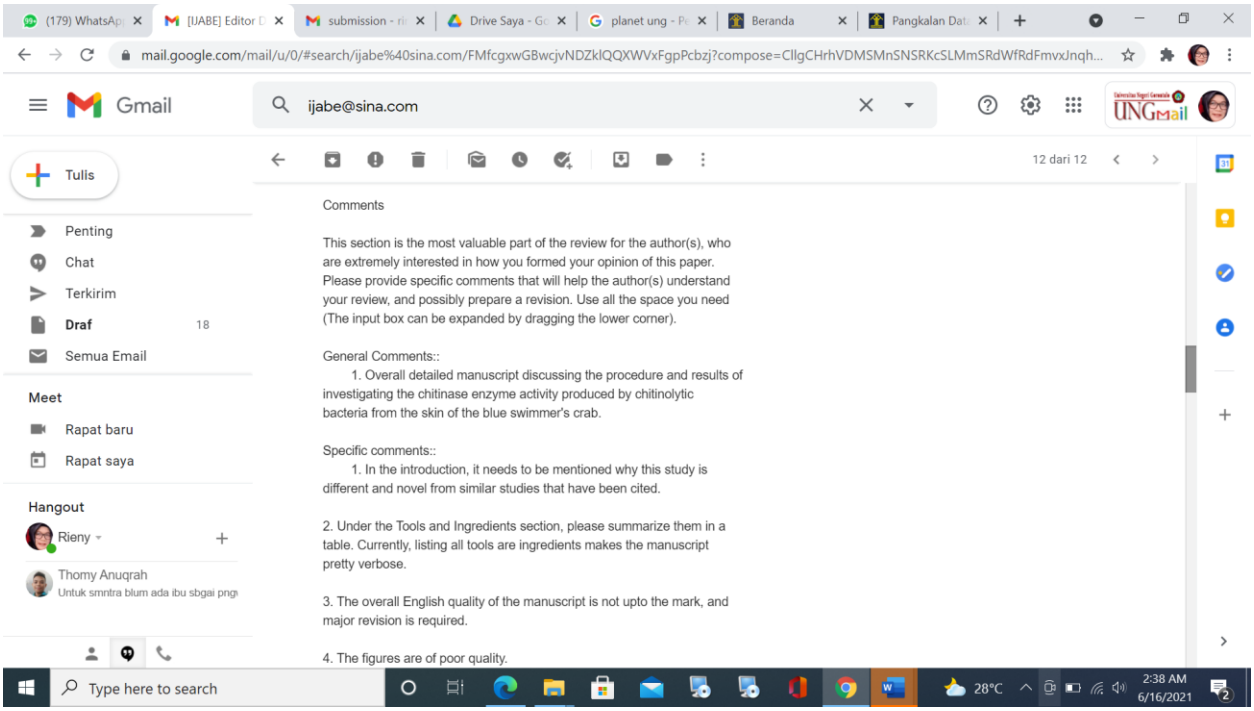
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Comments
This section is the most valuable part of the review for the author(s), who are extremely interested in how you formed your opinion of this paper. Please provide specific comments that will help the author(s) understand your review, and possibly prepare a revision. Use all the space you need (The input box can be expanded by dragging the lower corner).
General Comments::
1. Overall detailed manuscript discussing the procedure and results of investigating the chitinase enzyme activity produced by chitinolytic bacteria from the skin of the blue swimmer's crab.
Specific comments::
1. In the introduction, it needs to be mentioned why this study is different and novel from similar studies that have been cited.
2. Under the Tools and Ingredients section, please summarize them in a table. Currently, listing all tools are ingredients makes the manuscript pretty verbose.
3. The overall English quality of the manuscript is not upto the mark, and major revision is required.
4. The figures are of poor quality.
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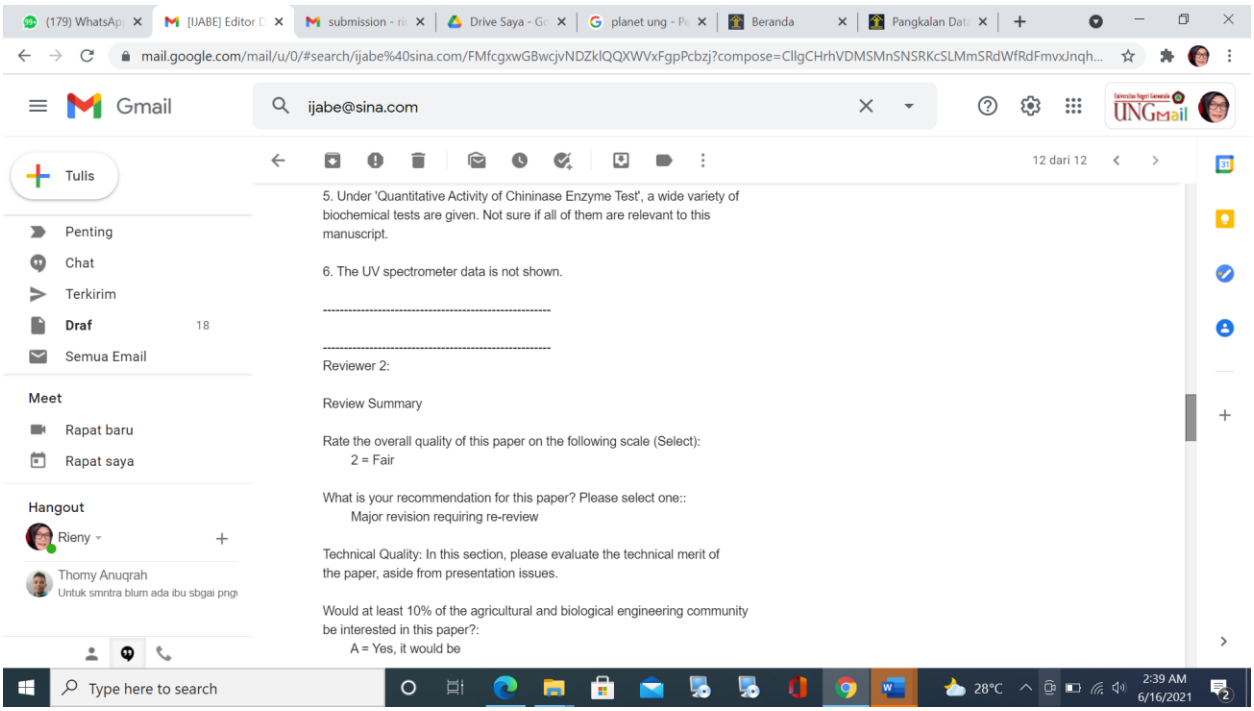
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5. Under 'Quantitative Activity of Chininase Enzyme Test', a wide variety of biochemical tests are given. Not sure if all of them are relevant to this manuscript.
6. The UV spectrometer data is not shown.

Reviewer 2:
Review Summary
Rate the overall quality of this paper on the following scale (Select):
2 = Fair
What is your recommendation for this paper? Please select one::
Major revision requiring re-review
Technical Quality: In this section, please evaluate the technical merit of the paper, aside from presentation issues.
Would at least 10% of the agricultural and biological engineering community be interested in this paper?:
A = Yes, it would be
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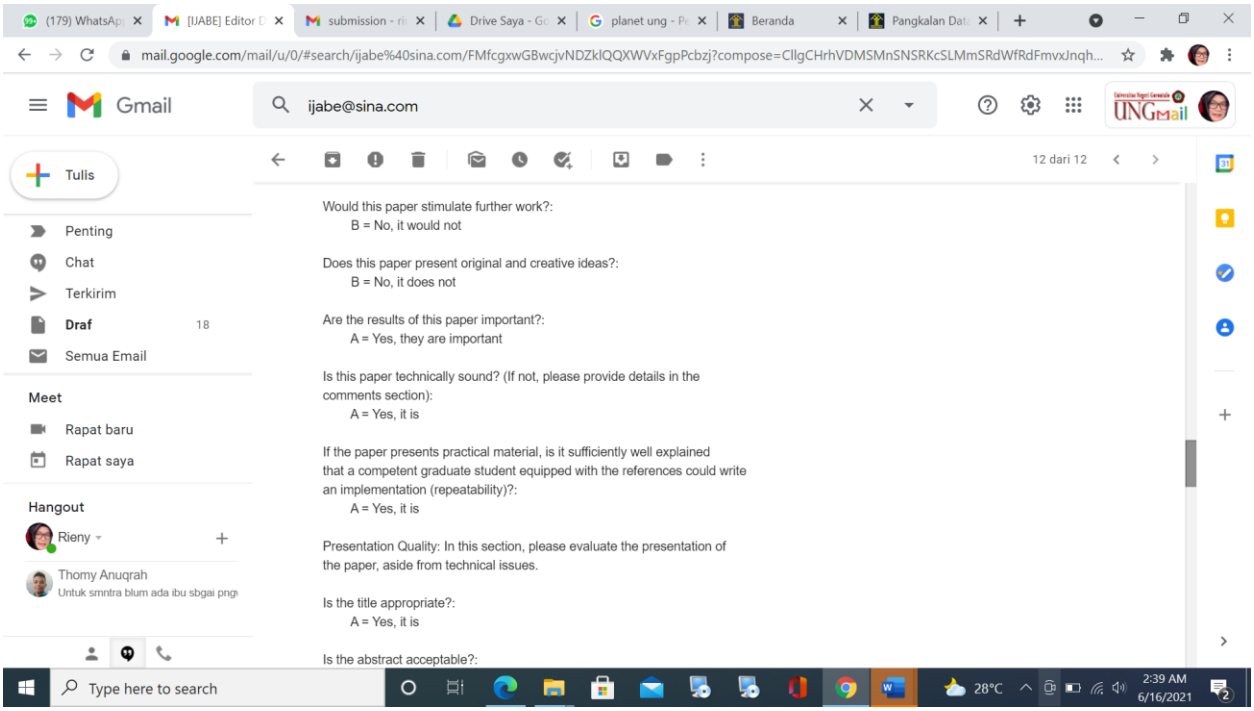
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Would this paper stimulate further work?:
B = No, it would not
Does this paper present original and creative ideas?:
B = No, it does not
Are the results of this paper important?:
A = Yes, they are important
Is this paper technically sound? (If not, please provide details in the comments section):
A = Yes, it is
If the paper presents practical material, is it sufficiently well explained that a competent graduate student equipped with the references could write an implementation (repeatability)?:
A = Yes, it is
Presentation Quality: In this section, please evaluate the presentation of the paper, aside from technical issues.
Is the title appropriate?:
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Is the abstract acceptable?:
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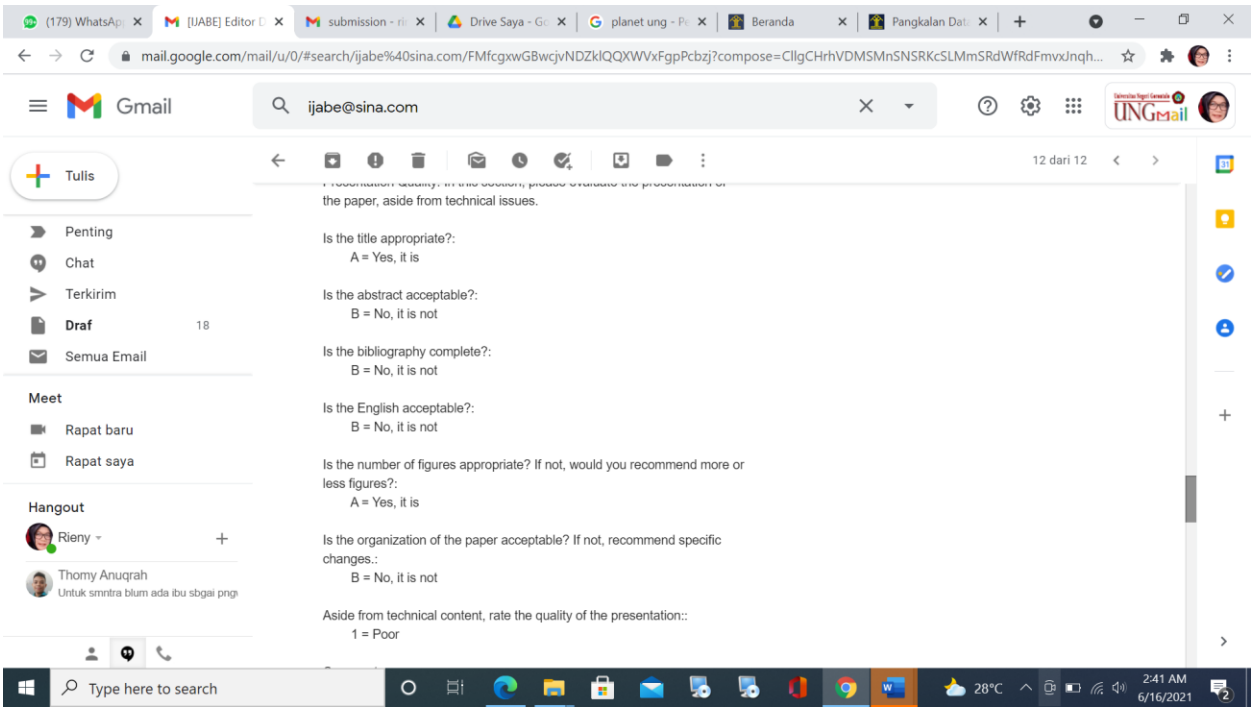
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Is the organization of the paper acceptable? If not, recommend specific changes.:
B = No, it is not
Aside from technical content, rate the quality of the presentation::
1 = Poor
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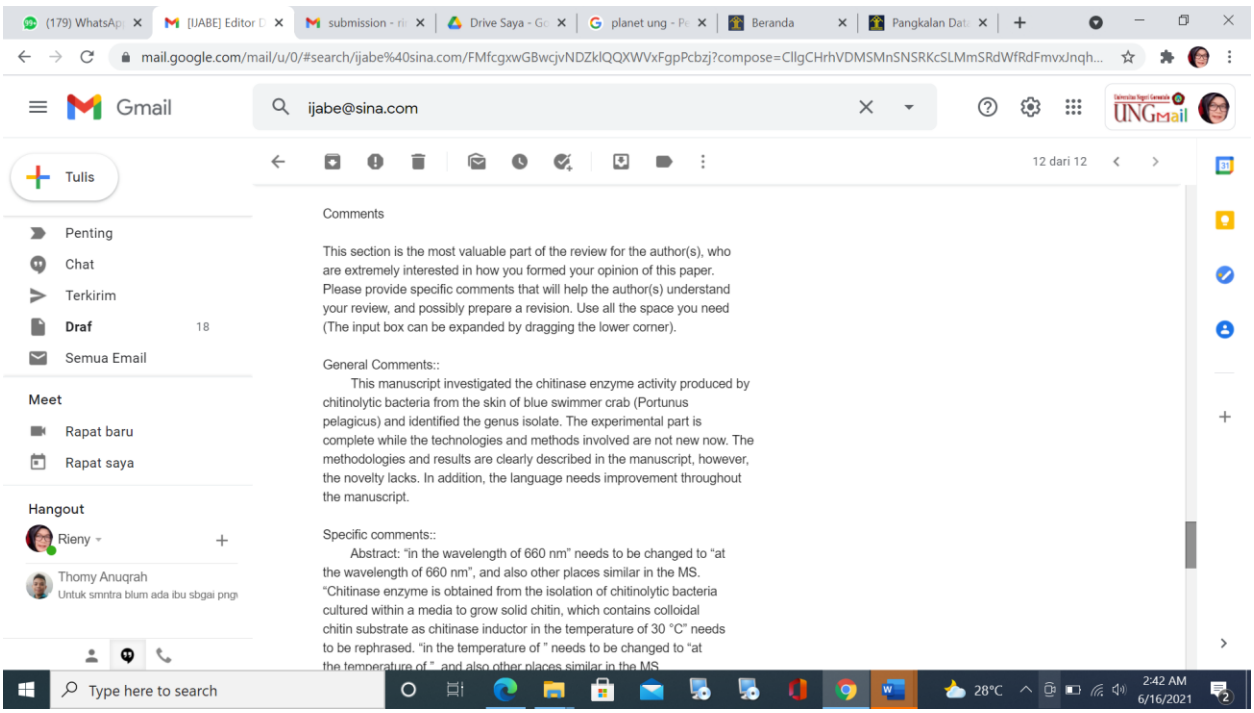
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Comments
This section is the most valuable part of the review for the author(s), who are extremely interested in how you formed your opinion of this paper. Please provide specific comments that will help the author(s) understand your review, and possibly prepare a revision. Use all the space you need (The input box can be expanded by dragging the lower corner).
General Comments::
This manuscript investigated the chitinase enzyme activity produced by chitinolytic bacteria from the skin of blue swimmer crab (Portunus pelagicus) and identified the genus isolate. The experimental part is complete while the technologies and methods involved are not new now. The methodologies and results are clearly described in the manuscript, however, the novelty lacks. In addition, the language needs improvement throughout the manuscript.
Specific comments::
Abstract: “in the wavelength of 660 nm” needs to be changed to “at the wavelength of 660 nm”, and also other places similar in the MS.
“Chitinase enzyme is obtained from the isolation of chitinolytic bacteria cultured within a media to grow solid chitin, which contains colloidal chitin substrate as chitinase inductor in the temperature of 30 °C” needs to be rephrased. “in the temperature of ” needs to be changed to “at
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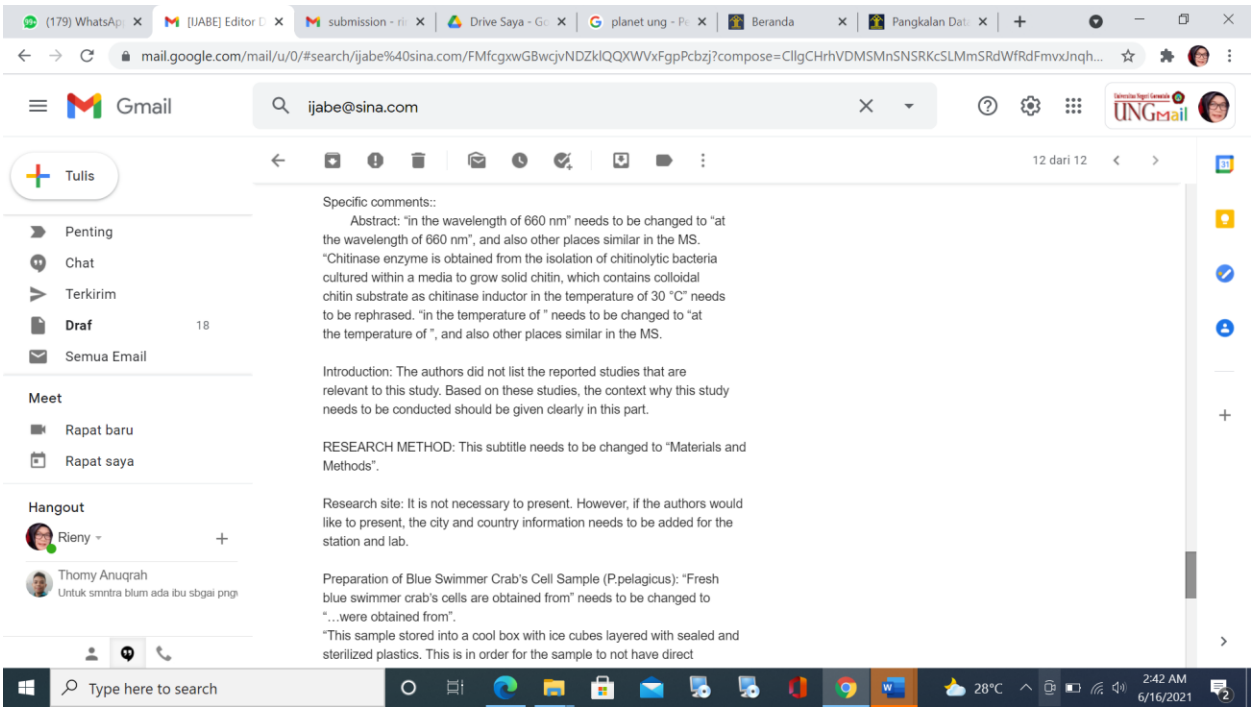


Specific comments::

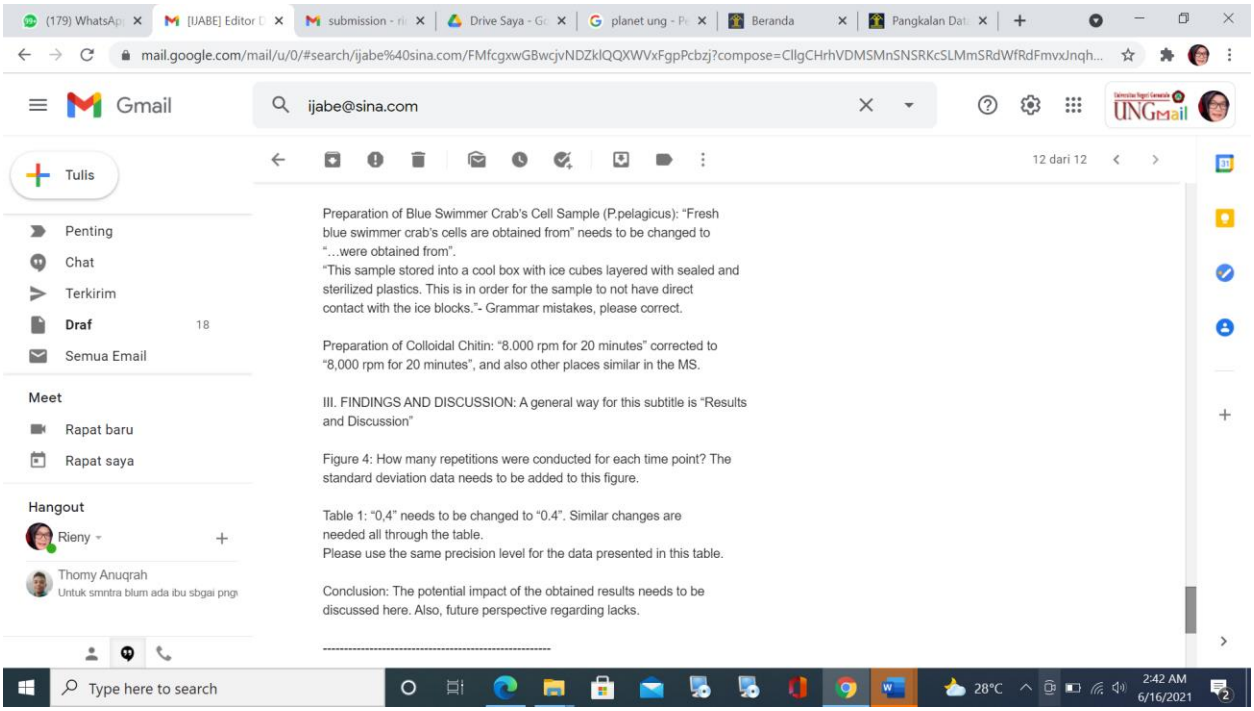
Abstract: "in the wavelength of 660 nm" needs to be changed to "at the wavelength of 660 nm", and also other places similar in the MS. "Chitinase enzyme is obtained from the isolation of chitinolytic bacteria cultured within a media to grow solid chitin, which contains colloidal chitin substrate as chitinase inductor in the temperature of 30 °C" needs to be rephrased. "in the temperature of " needs to be changed to "at the temperature of ", and also other places similar in the MS.

Introduction: The authors did not list the reported studies that are relevant to this study. Based on these studies, the context why this study needs to be conducted should be given clearly in this part.

RESEARCH METHOD: This subtitle needs to be changed to "Materials and Methods".

Research site: It is not necessary to present. However, if the authors would like to present, the city and country information needs to be added for the station and lab.

Preparation of Blue Swimmer Crab's Cell Sample (P.pelagicus): "Fresh blue swimmer crab's cells are obtained from" needs to be changed to "…were obtained from".
"This sample stored into a cool box with ice cubes layered with sealed and sterilized plastics. This is in order for the sample to not have direct



Preparation of Blue Swimmer Crab's Cell Sample (P.pelagicus): "Fresh blue swimmer crab's cells are obtained from" needs to be changed to "…were obtained from".
"This sample stored into a cool box with ice cubes layered with sealed and sterilized plastics. This is in order for the sample to not have direct contact with the ice blocks."- Grammar mistakes, please correct.

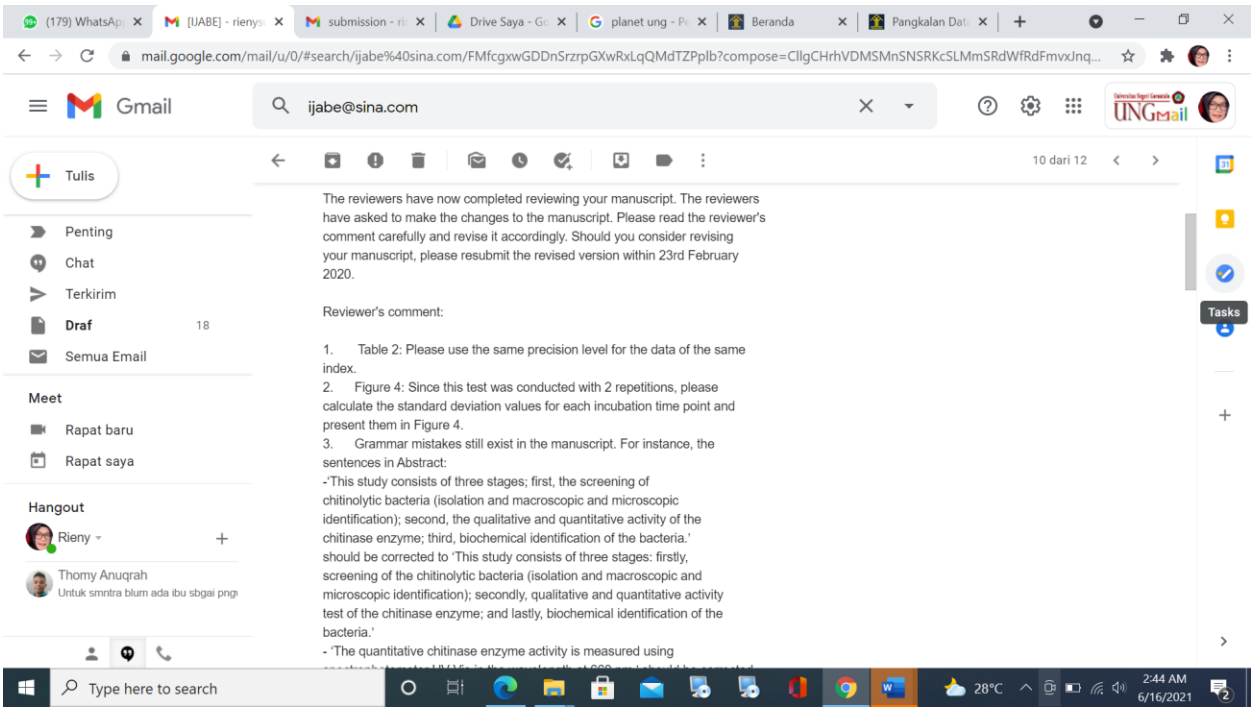
Preparation of Colloidal Chitin: "8.000 rpm for 20 minutes" corrected to "8,000 rpm for 20 minutes", and also other places similar in the MS.

III. FINDINGS AND DISCUSSION: A general way for this subtitle is "Results and Discussion"

Figure 4: How many repetitions were conducted for each time point? The standard deviation data needs to be added to this figure.

Table 1: "0,4" needs to be changed to "0.4". Similar changes are needed all through the table.
Please use the same precision level for the data presented in this table.

Conclusion: The potential impact of the obtained results needs to be discussed here. Also, future perspective regarding lacks.



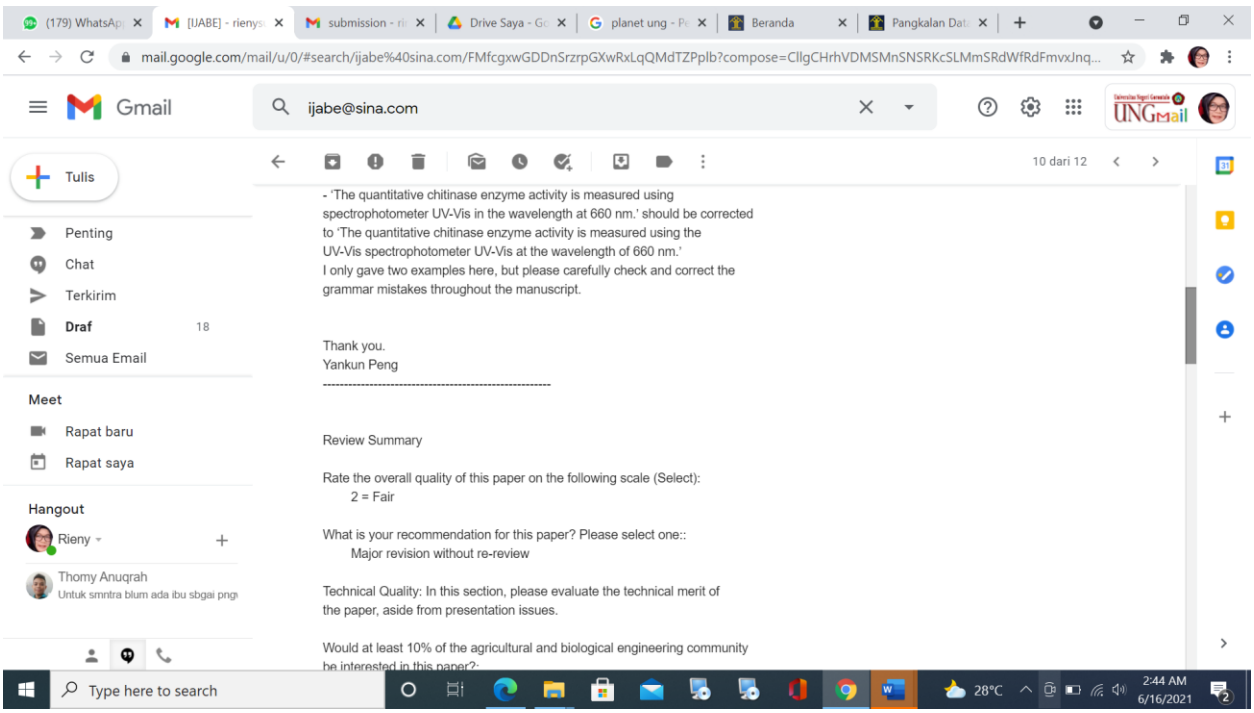
The reviewers have now completed reviewing your manuscript. The reviewers have asked to make the changes to the manuscript. Please read the reviewer's comment carefully and revise it accordingly. Should you consider revising your manuscript, please resubmit the revised version within 23rd February 2020.

Reviewer's comment:

1. Table 2: Please use the same precision level for the data of the same index.
2. Figure 4: Since this test was conducted with 2 repetitions, please calculate the standard deviation values for each incubation time point and present them in Figure 4.
3. Grammar mistakes still exist in the manuscript. For instance, the sentences in Abstract:

-'This study consists of three stages; first, the screening of chitinolytic bacteria (isolation and macroscopic and microscopic identification); second, the qualitative and quantitative activity of the chitinase enzyme; third, biochemical identification of the bacteria.' should be corrected to 'This study consists of three stages: firstly, screening of the chitinolytic bacteria (isolation and macroscopic and microscopic identification); secondly, qualitative and quantitative activity test of the chitinase enzyme; and lastly, biochemical identification of the bacteria.'

- 'The quantitative chitinase enzyme activity is measured using



- 'The quantitative chitinase enzyme activity is measured using spectrophotometer UV-Vis in the wavelength at 660 nm.' should be corrected to 'The quantitative chitinase enzyme activity is measured using the UV-Vis spectrophotometer UV-Vis at the wavelength of 660 nm.'
I only gave two examples here, but please carefully check and correct the grammar mistakes throughout the manuscript.

Thank you.
Yankun Peng

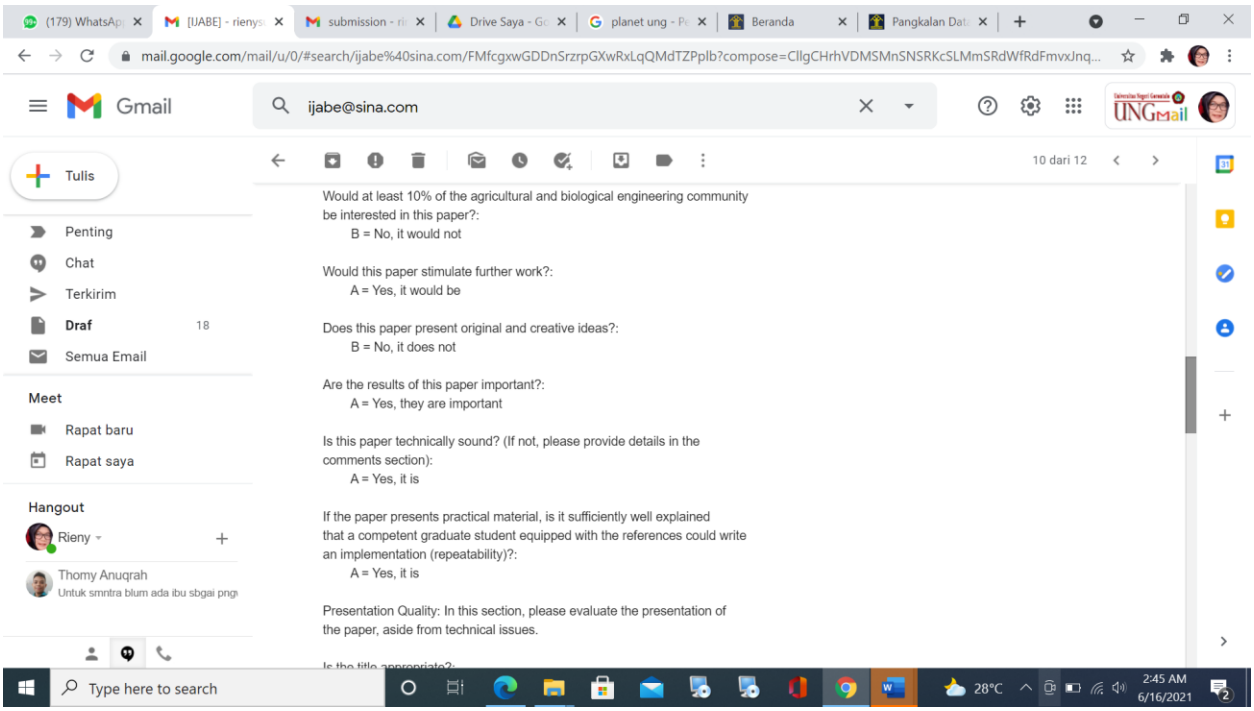
Review Summary

Rate the overall quality of this paper on the following scale (Select):
2 = Fair

What is your recommendation for this paper? Please select one::
Major revision without re-review

Technical Quality: In this section, please evaluate the technical merit of the paper, aside from presentation issues.

Would at least 10% of the agricultural and biological engineering community be interested in this paper?: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Would at least 10% of the agricultural and biological engineering community be interested in this paper?:
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Presentation Quality: In this section, please evaluate the presentation of the paper, aside from technical issues.
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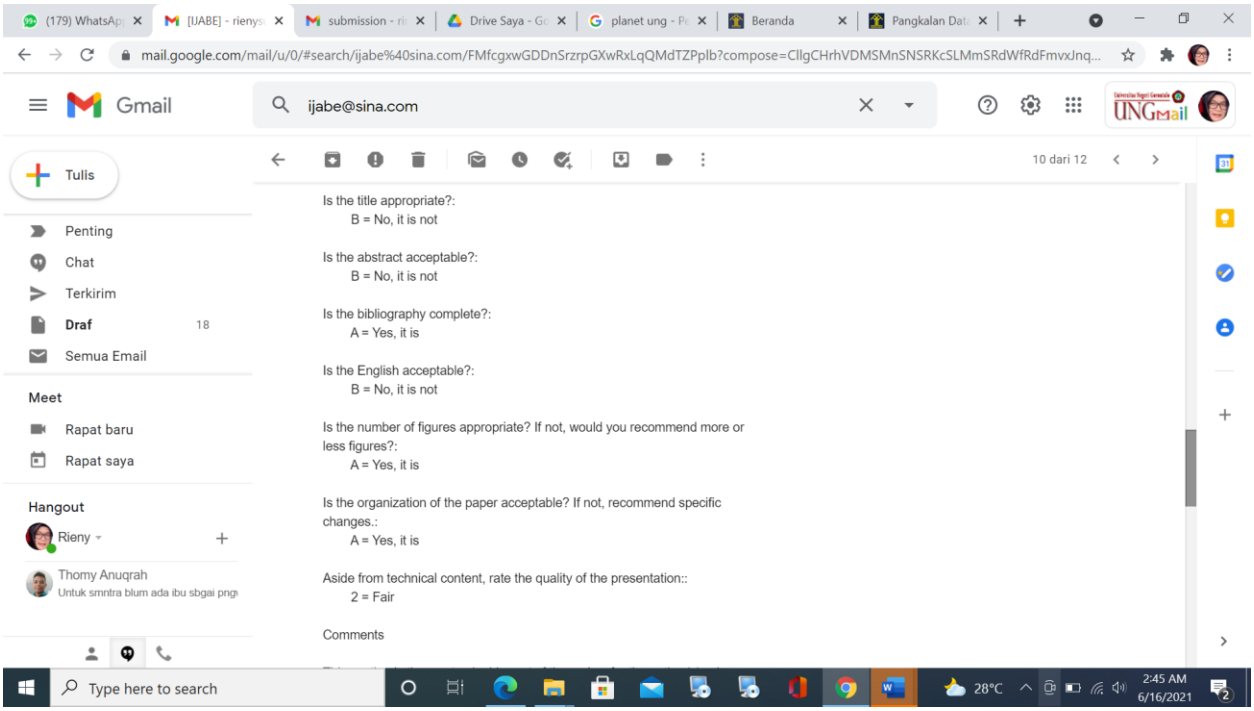
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A = Yes, it is
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2 = Fair
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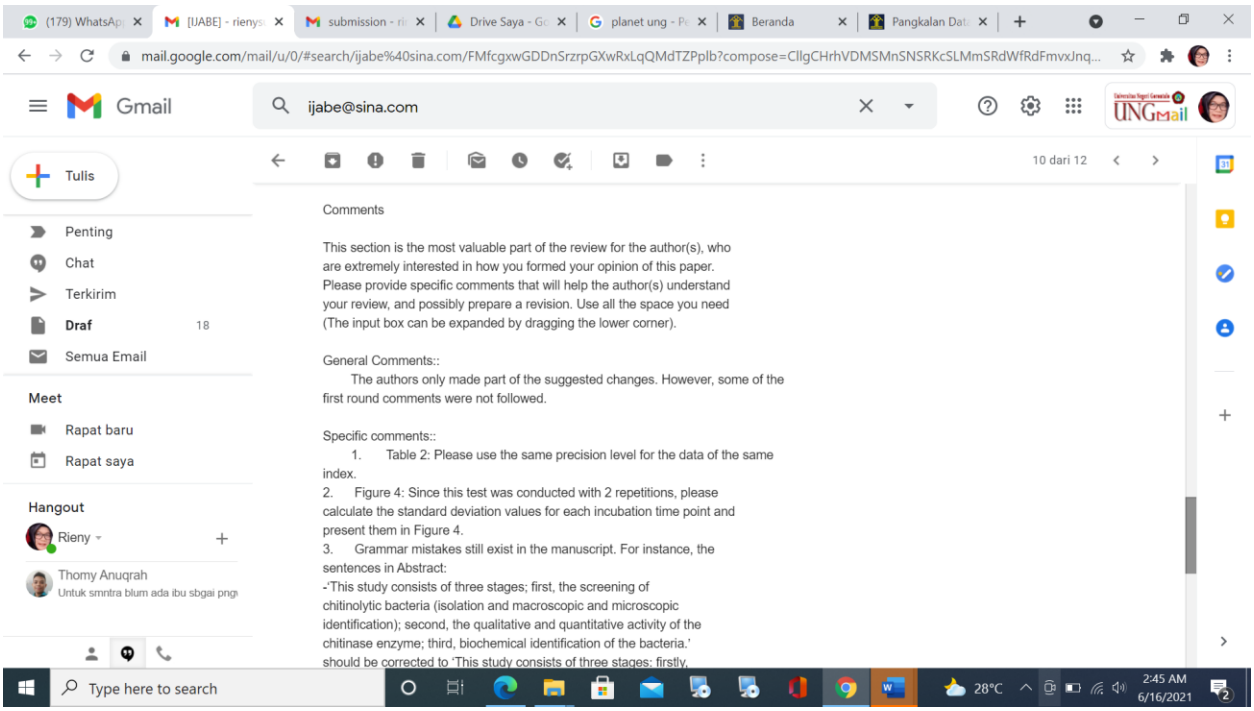

Comments

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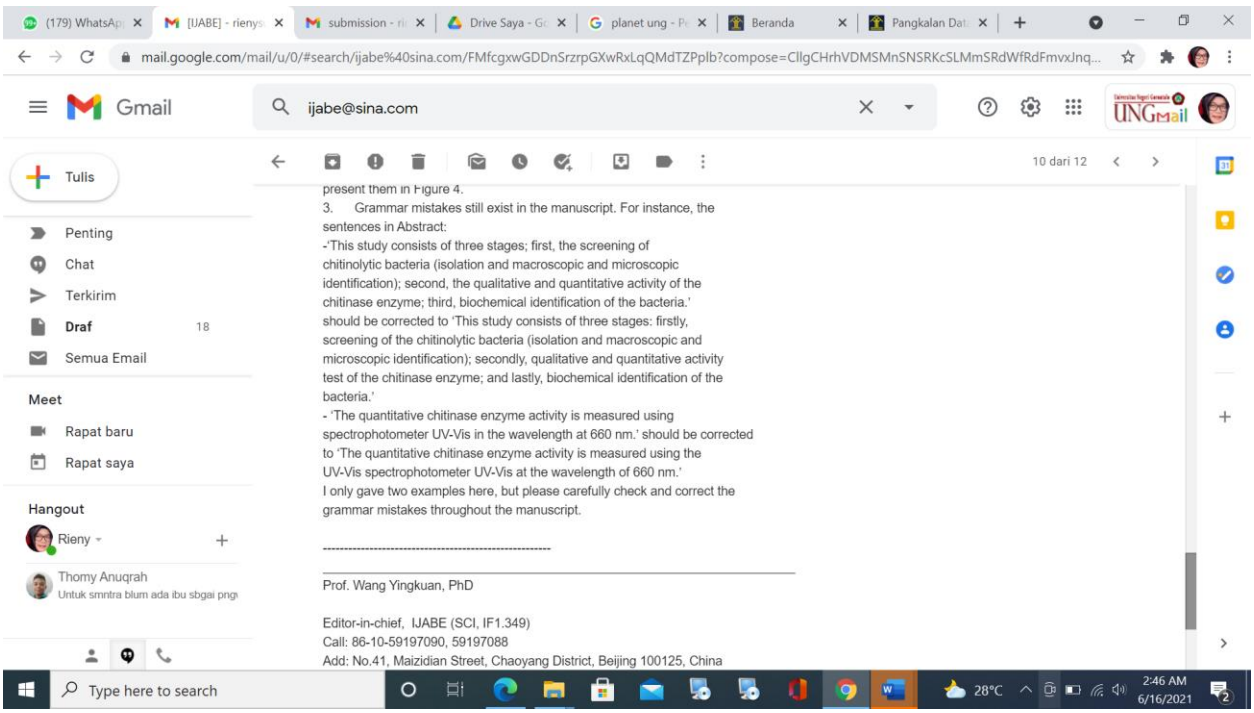
General Comments::
The authors only made part of the suggested changes. However, some of the first round comments were not followed.

Specific comments::
1. Table 2: Please use the same precision level for the data of the same index.
2. Figure 4: Since this test was conducted with 2 repetitions, please calculate the standard deviation values for each incubation time point and present them in Figure 4.
3. Grammar mistakes still exist in the manuscript. For instance, the sentences in Abstract:
-'This study consists of three stages; first, the screening of chitinolytic bacteria (isolation and macroscopic and microscopic identification); second, the qualitative and quantitative activity of the chitinase enzyme; third, biochemical identification of the bacteria.' should be corrected to 'This study consists of three stages: firstly,



present them in Figure 4.
3. Grammar mistakes still exist in the manuscript. For instance, the sentences in Abstract:
-'This study consists of three stages; first, the screening of chitinolytic bacteria (isolation and macroscopic and microscopic identification); second, the qualitative and quantitative activity of the chitinase enzyme; third, biochemical identification of the bacteria.' should be corrected to 'This study consists of three stages: firstly, screening of the chitinolytic bacteria (isolation and macroscopic and microscopic identification); secondly, qualitative and quantitative activity test of the chitinase enzyme; and lastly, biochemical identification of the bacteria.'
- 'The quantitative chitinase enzyme activity is measured using spectrophotometer UV-Vis in the wavelength at 660 nm.' should be corrected to 'The quantitative chitinase enzyme activity is measured using the UV-Vis spectrophotometer UV-Vis at the wavelength of 660 nm.'
I only gave two examples here, but please carefully check and correct the grammar mistakes throughout the manuscript.

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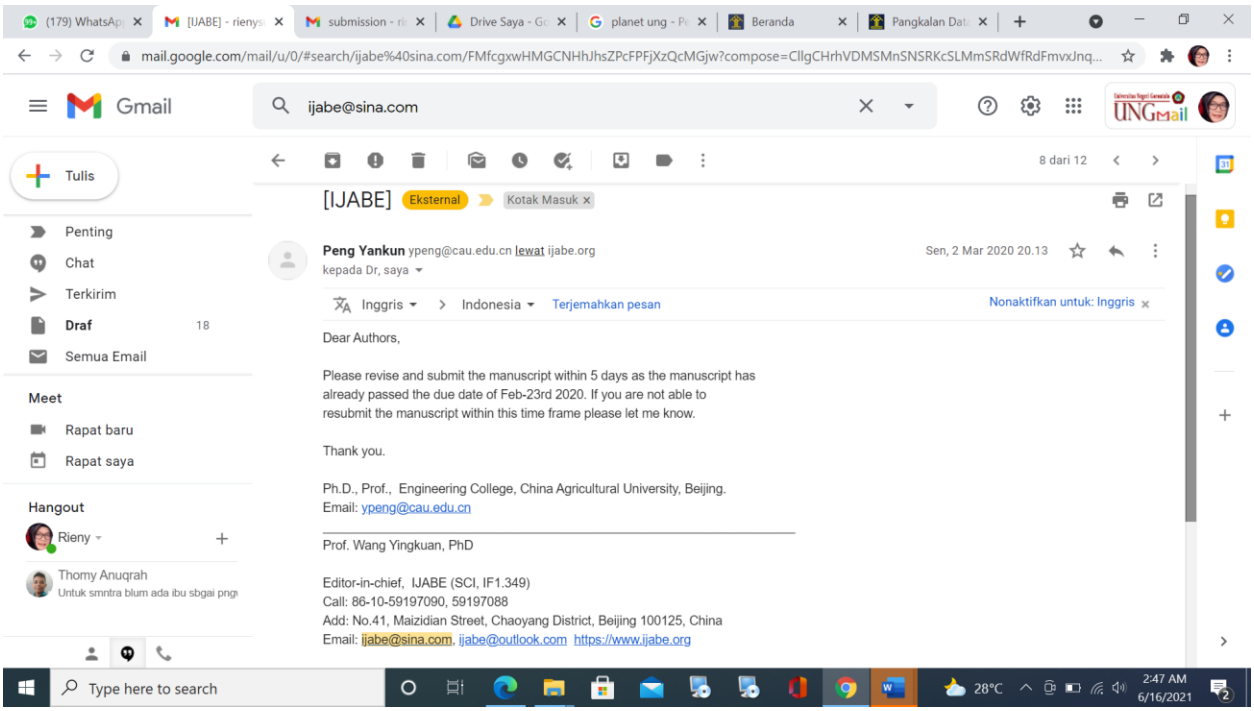

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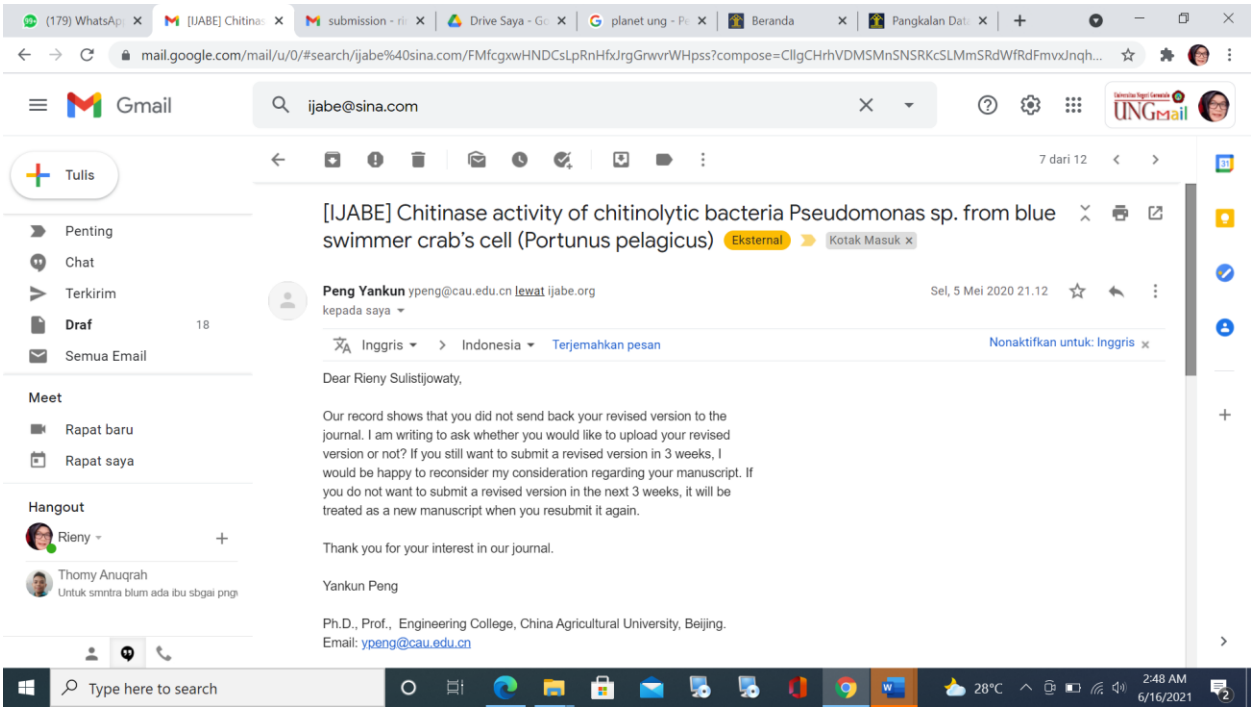

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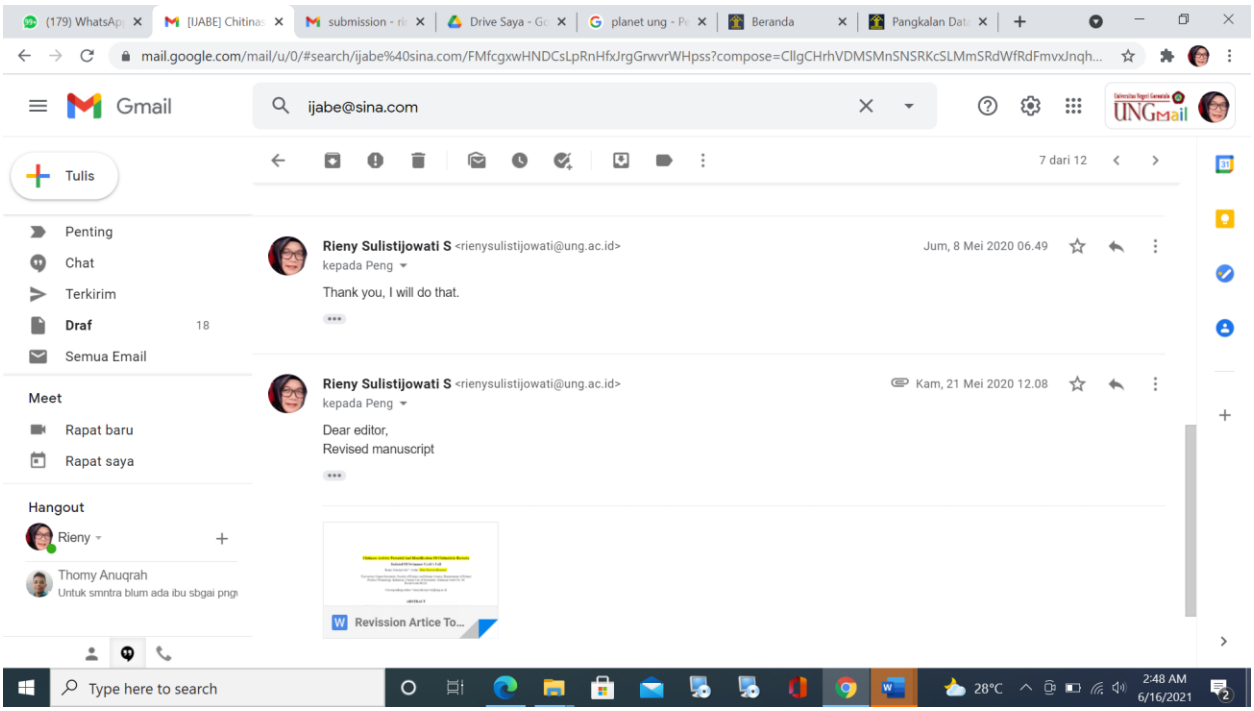
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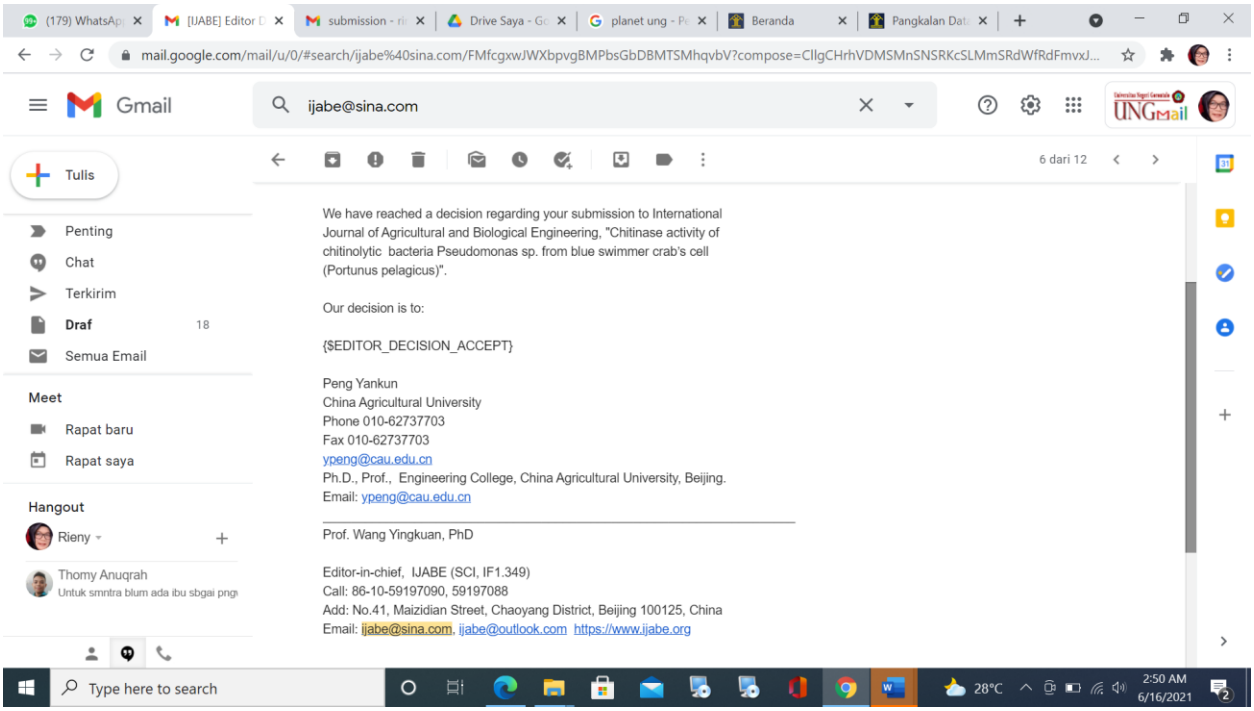
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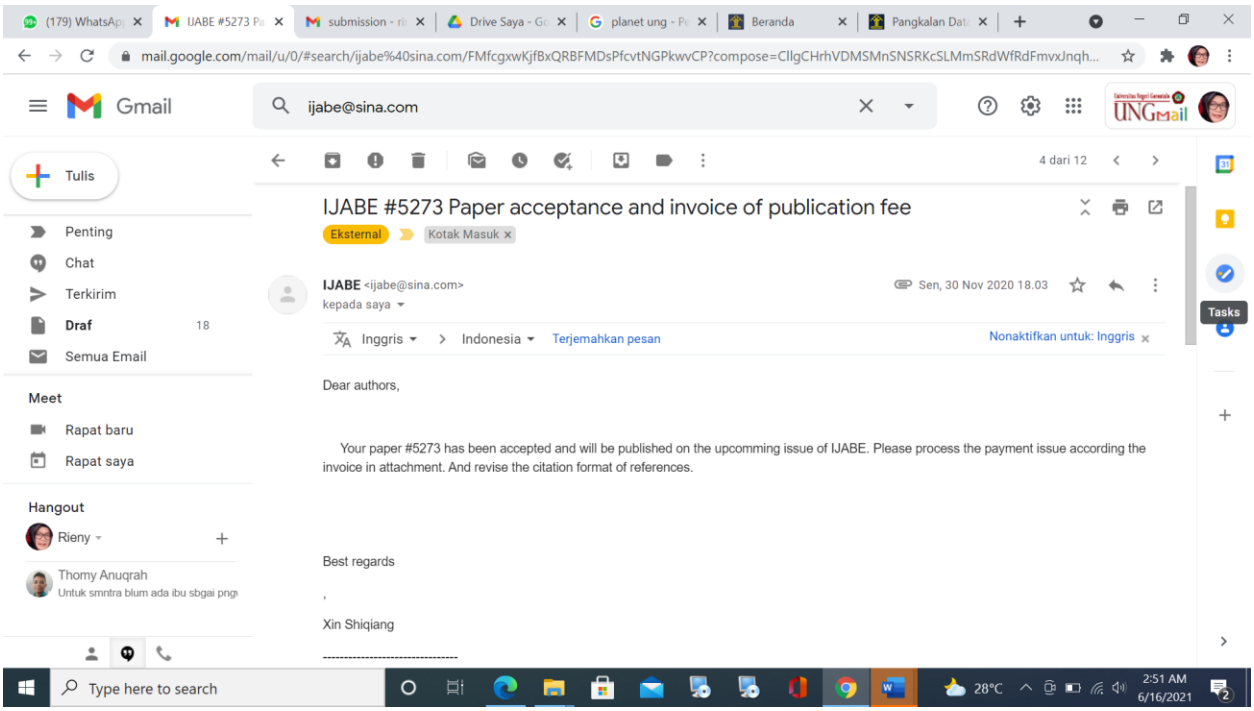
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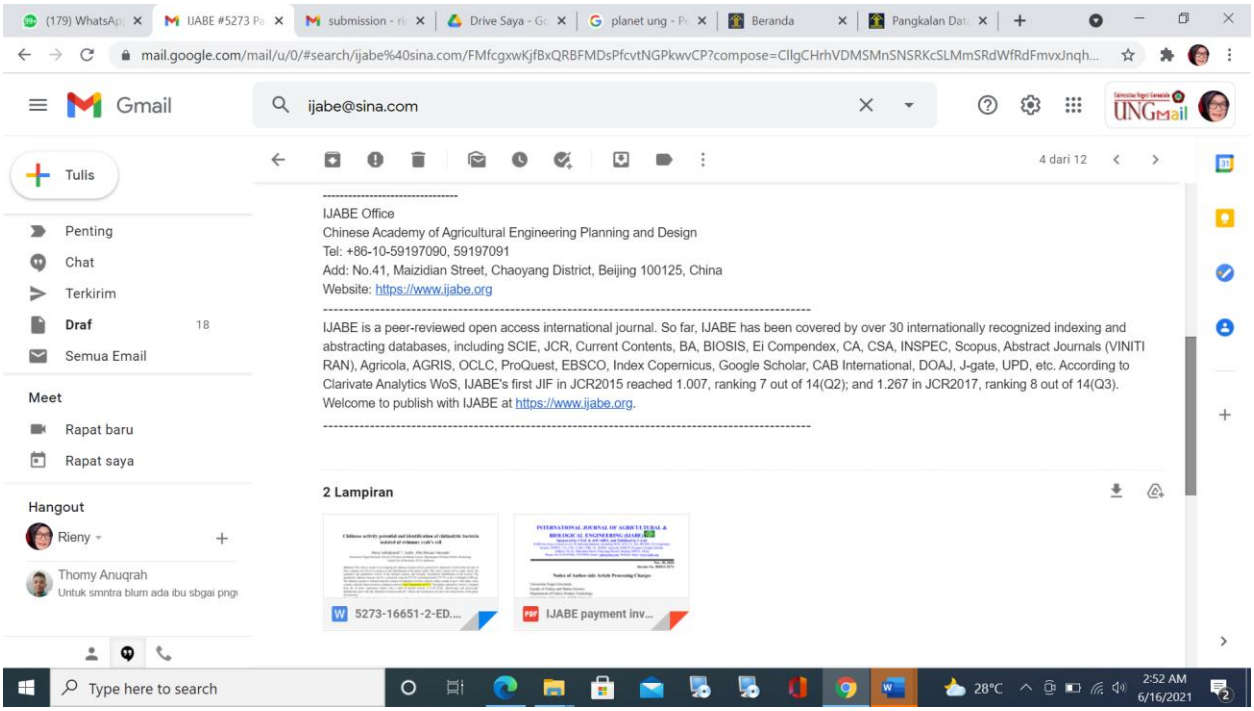
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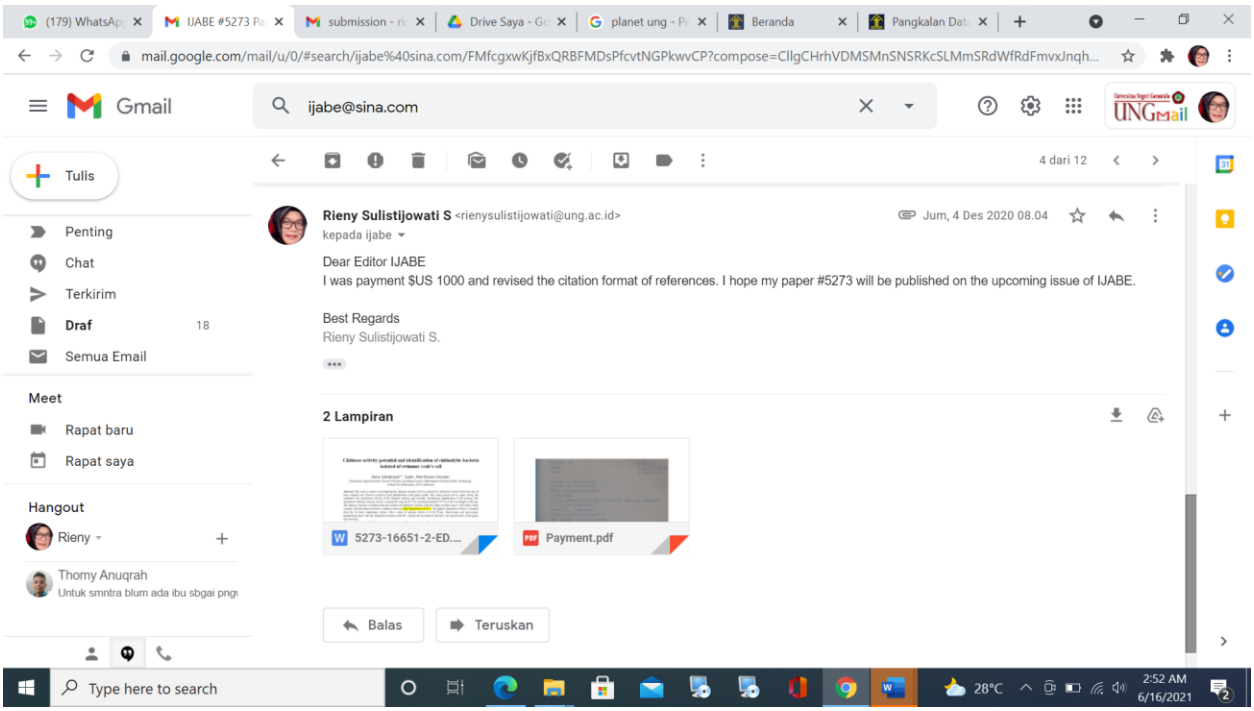
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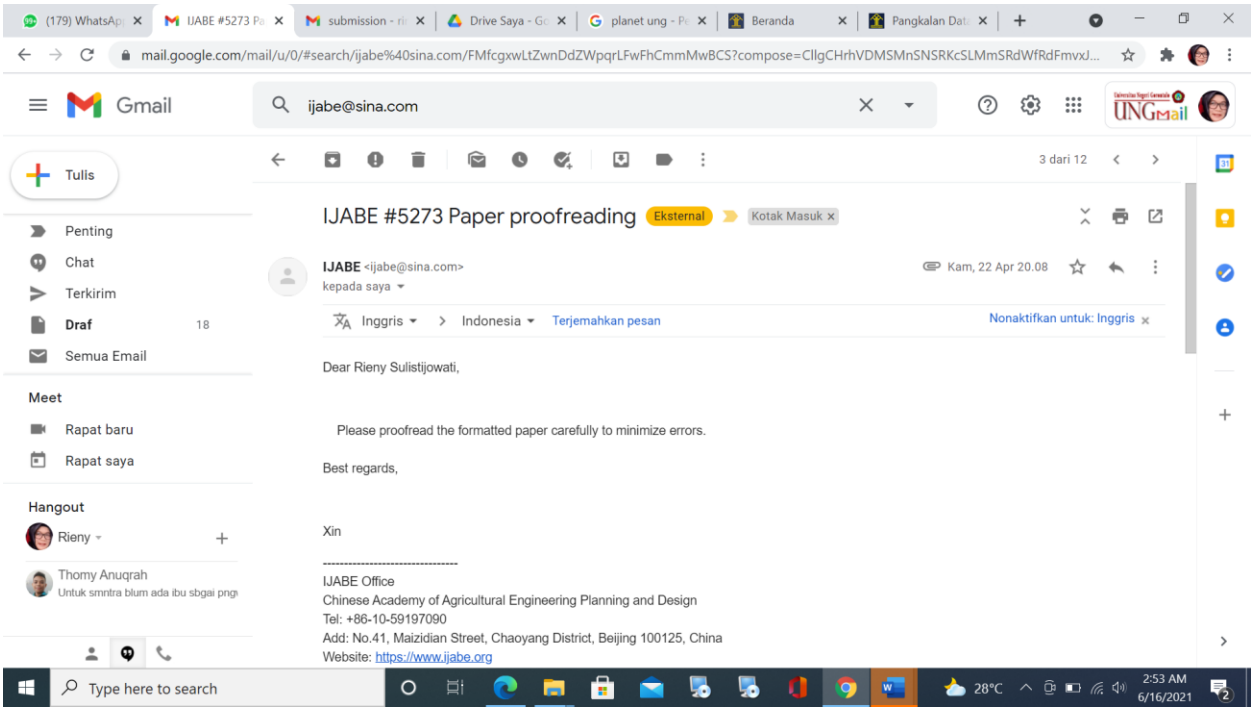
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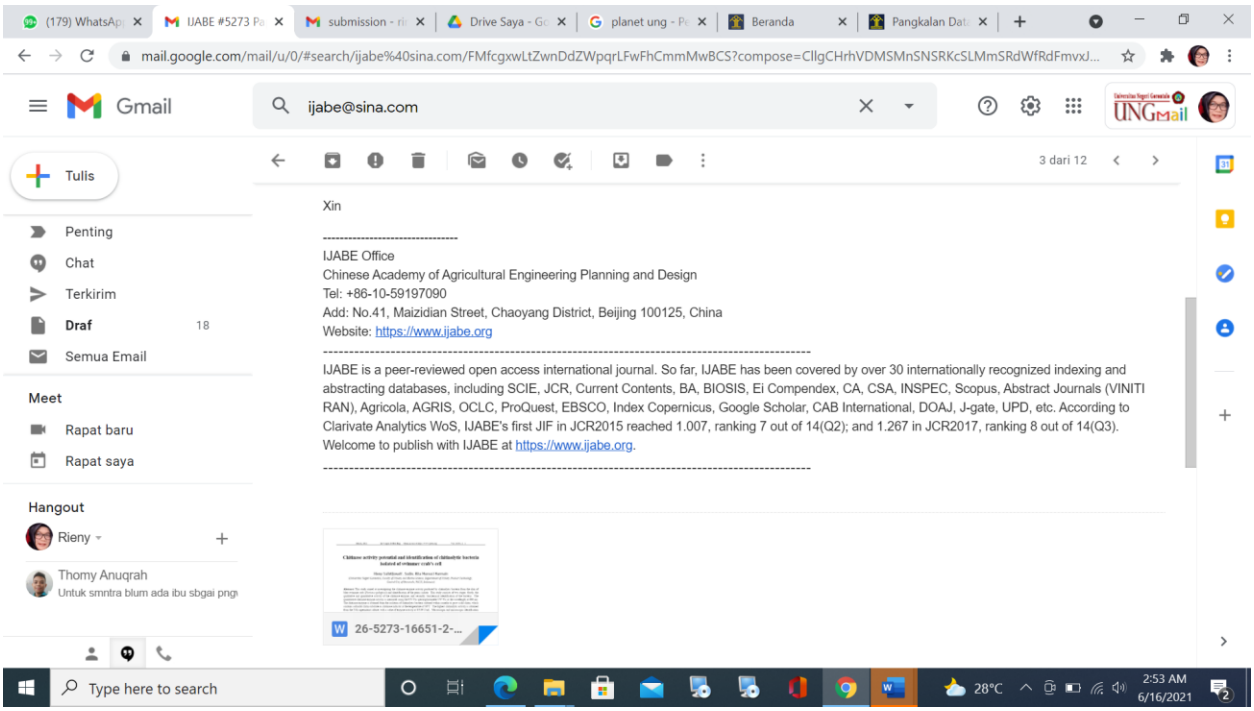
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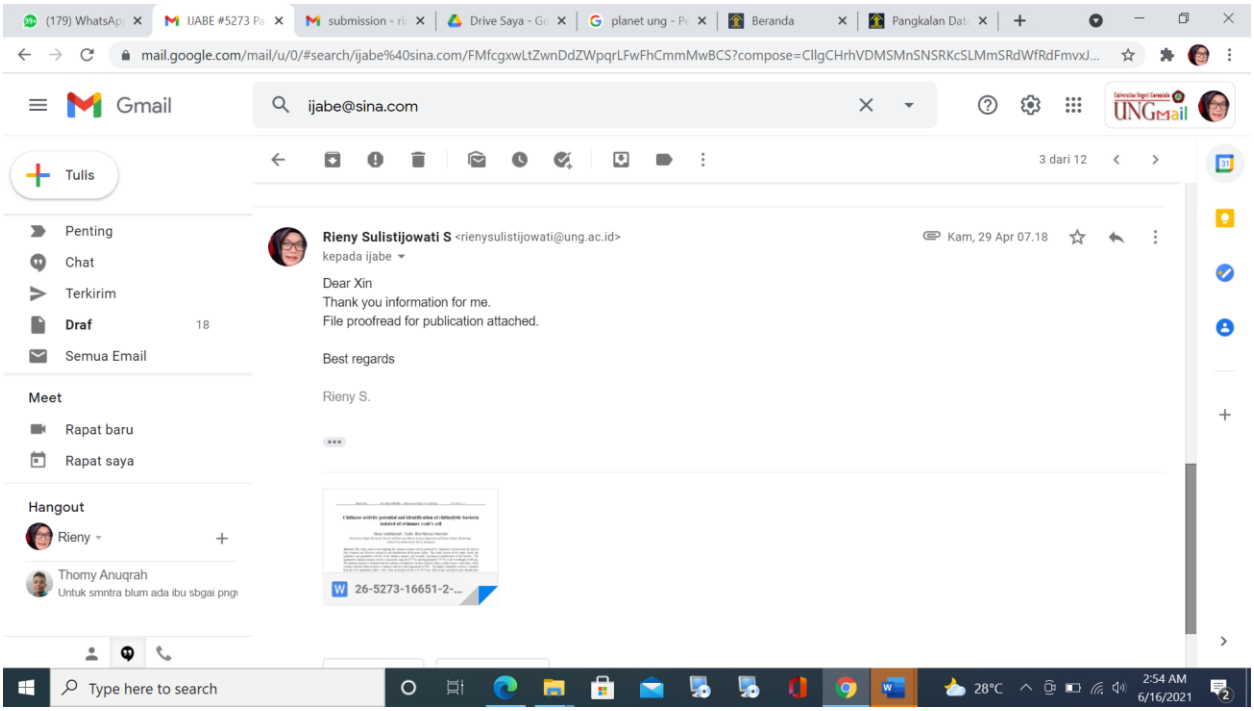
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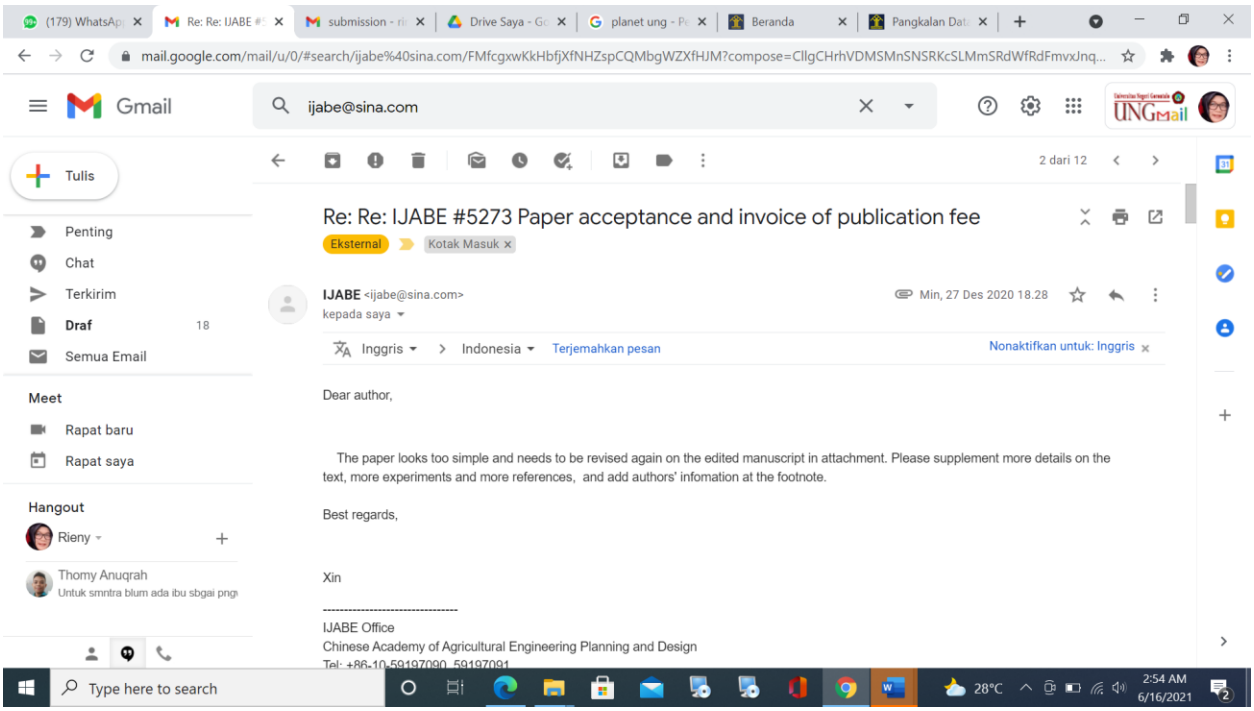

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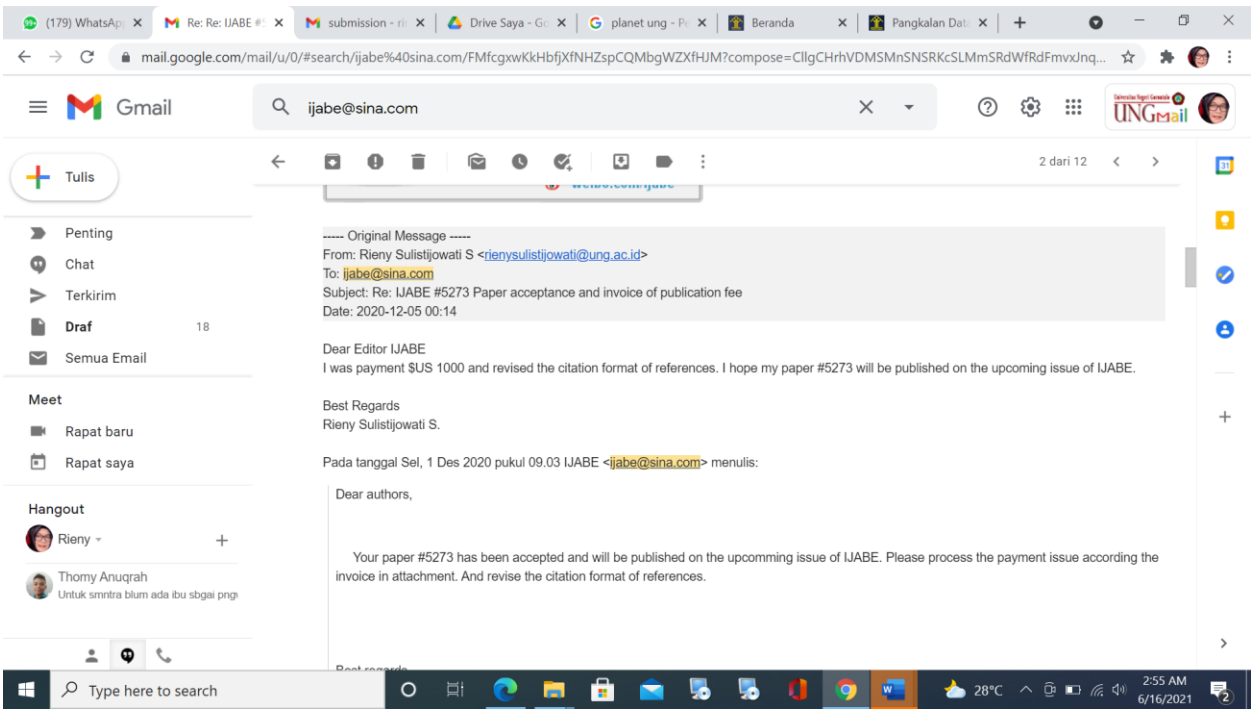

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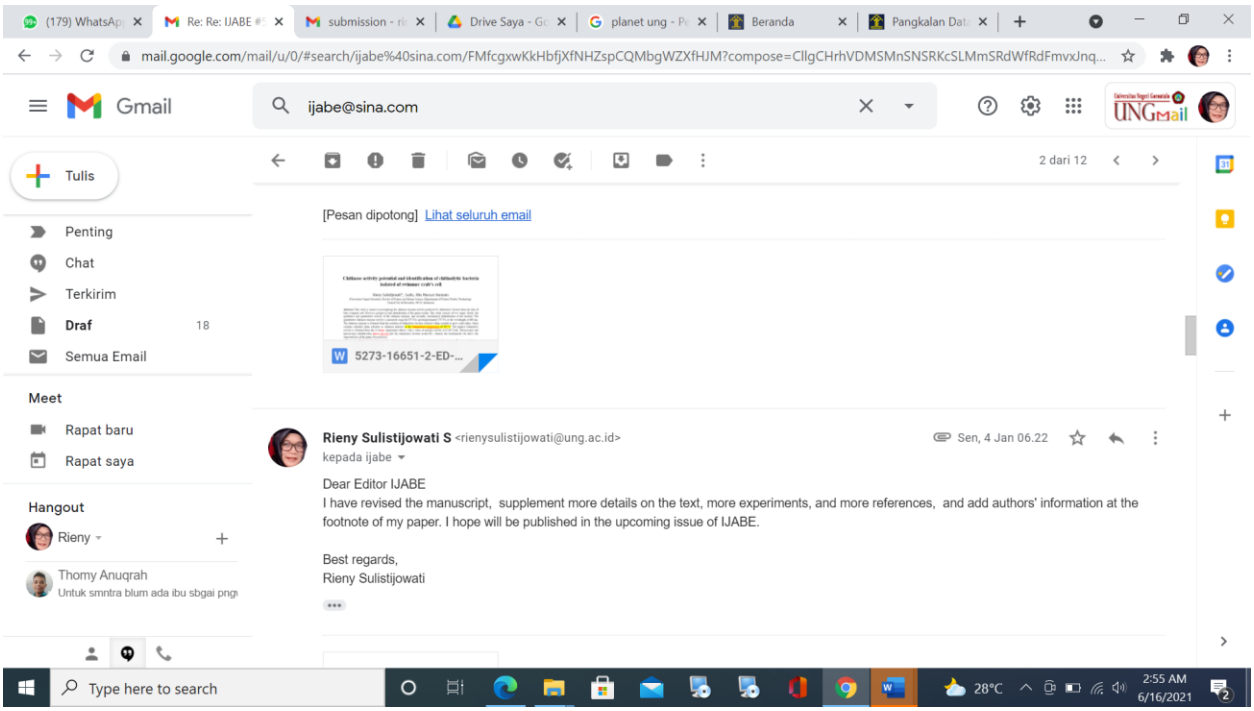
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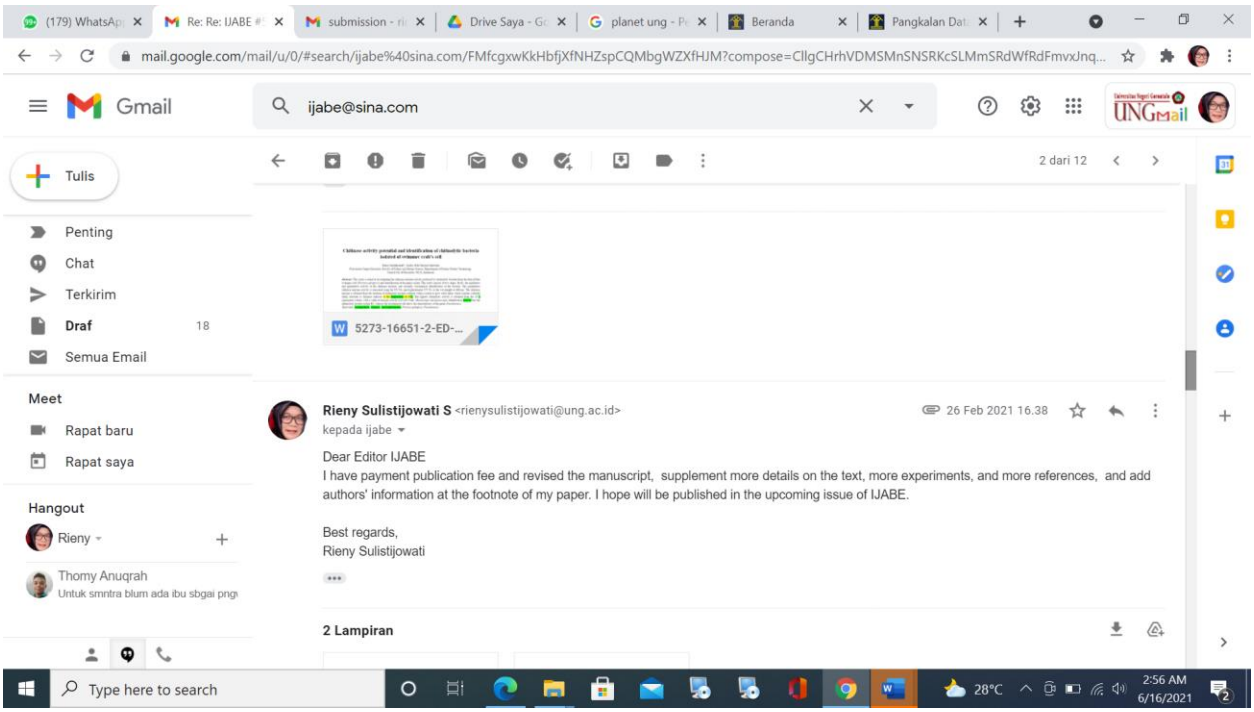
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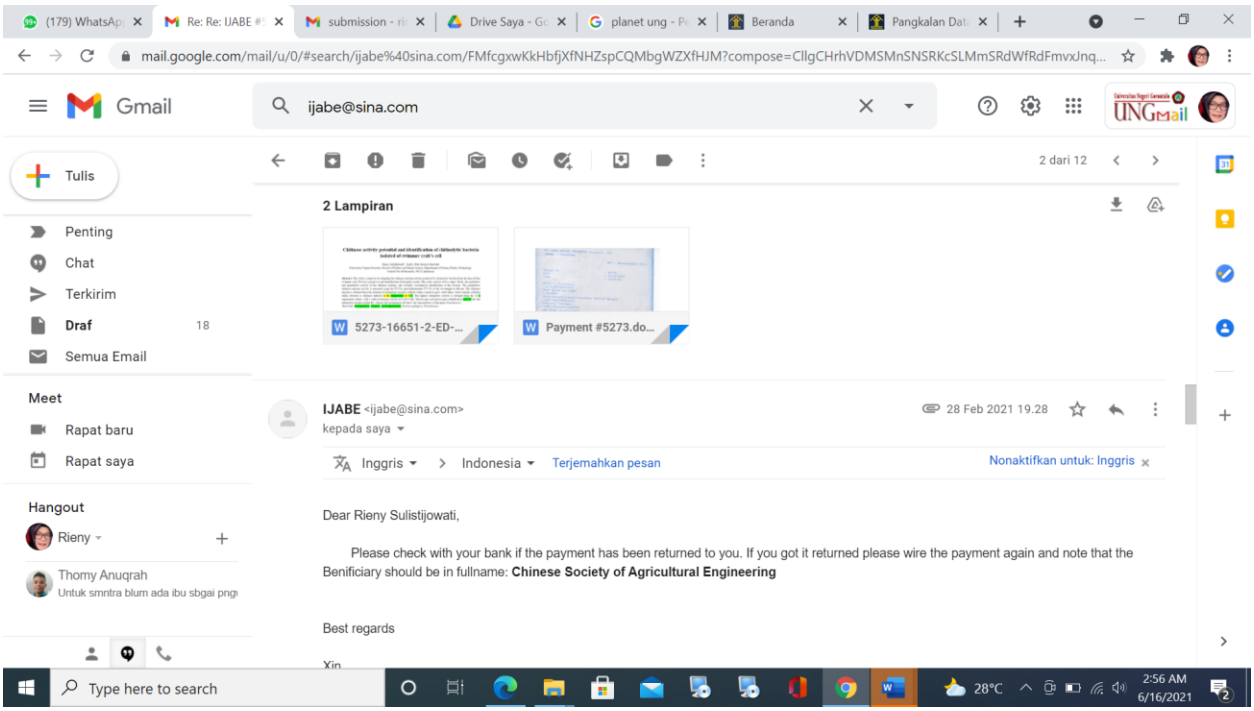
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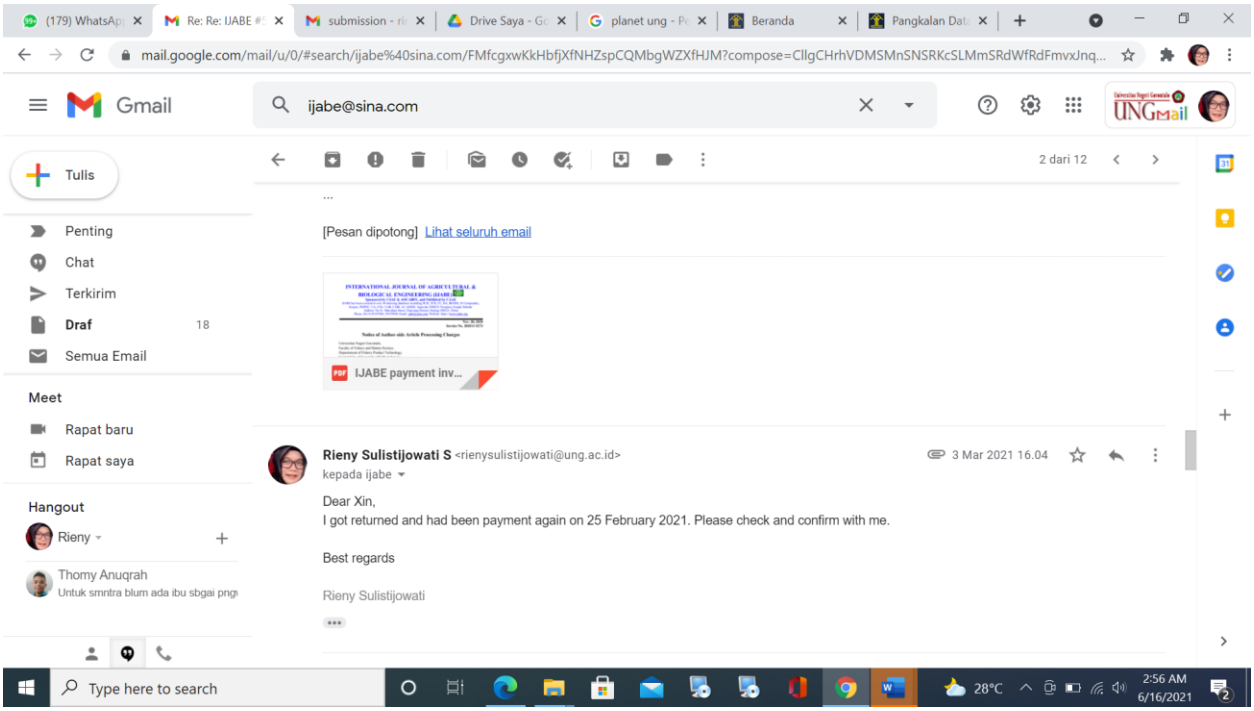
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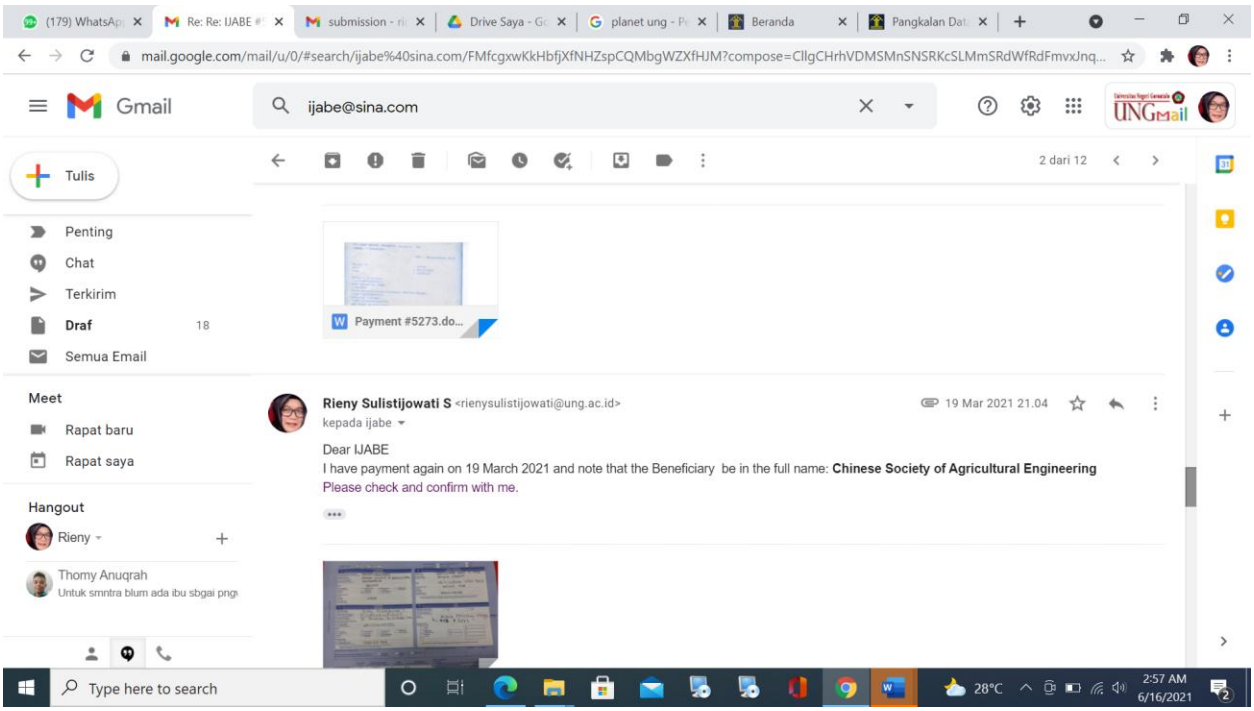
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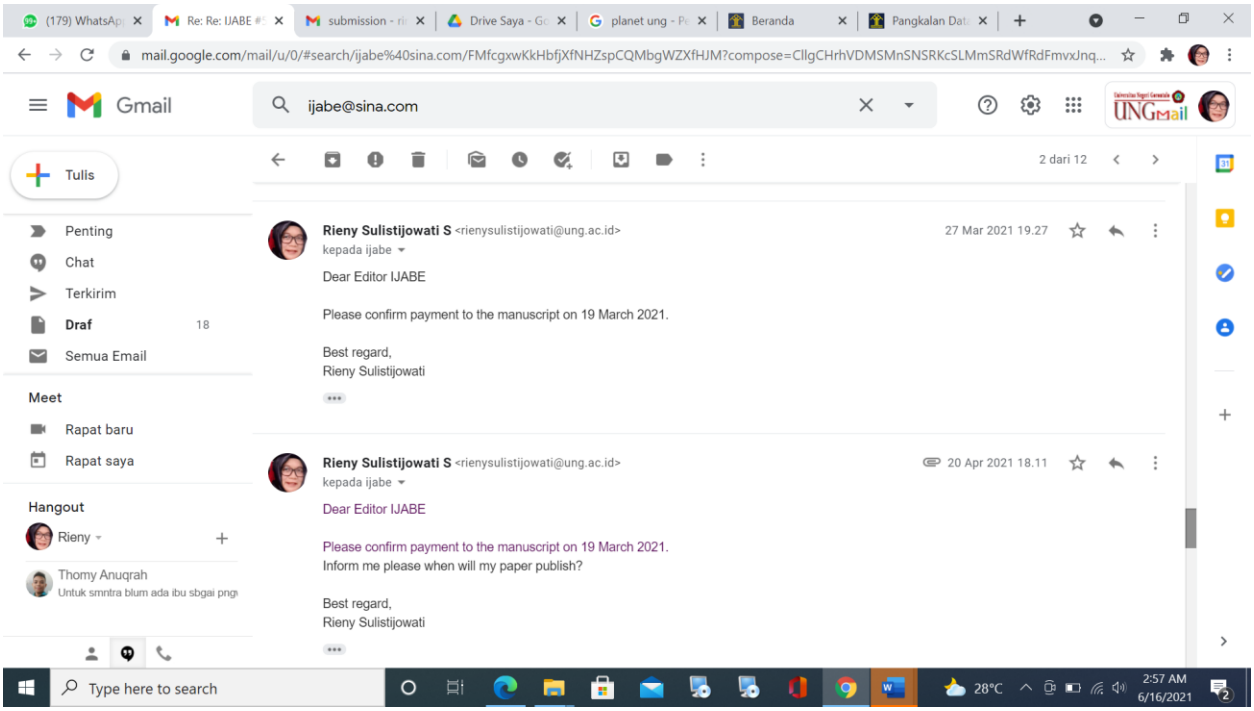
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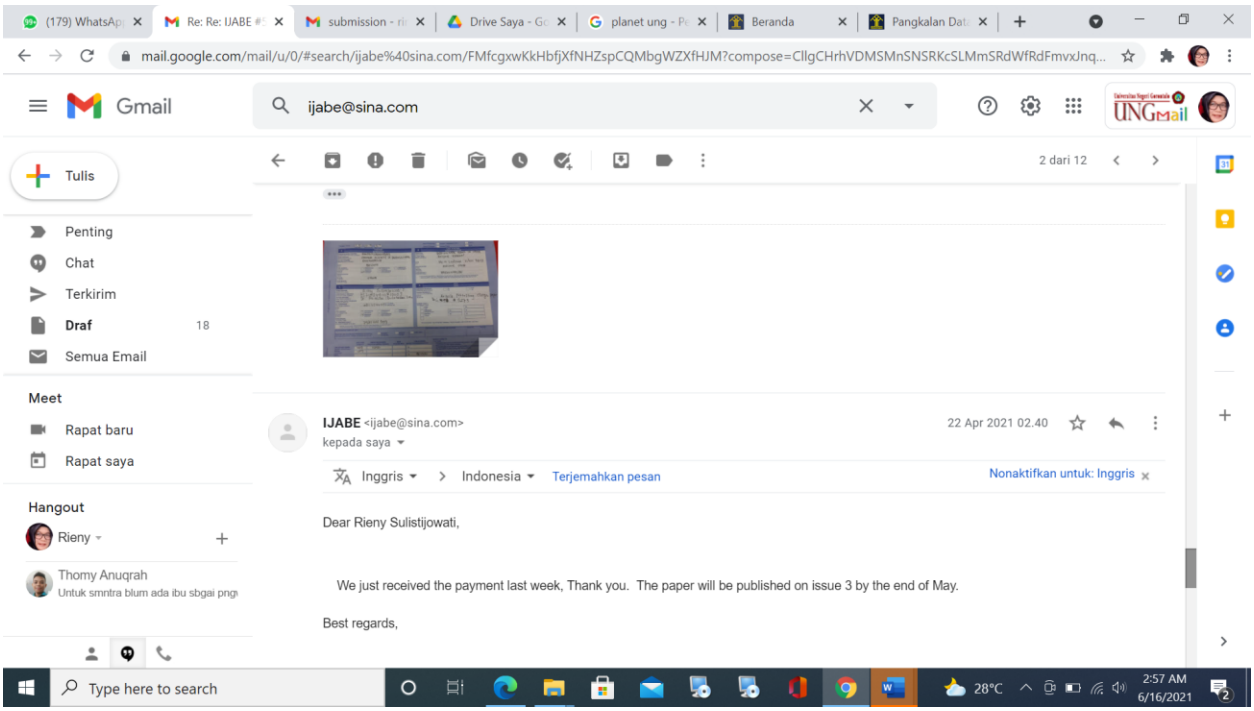
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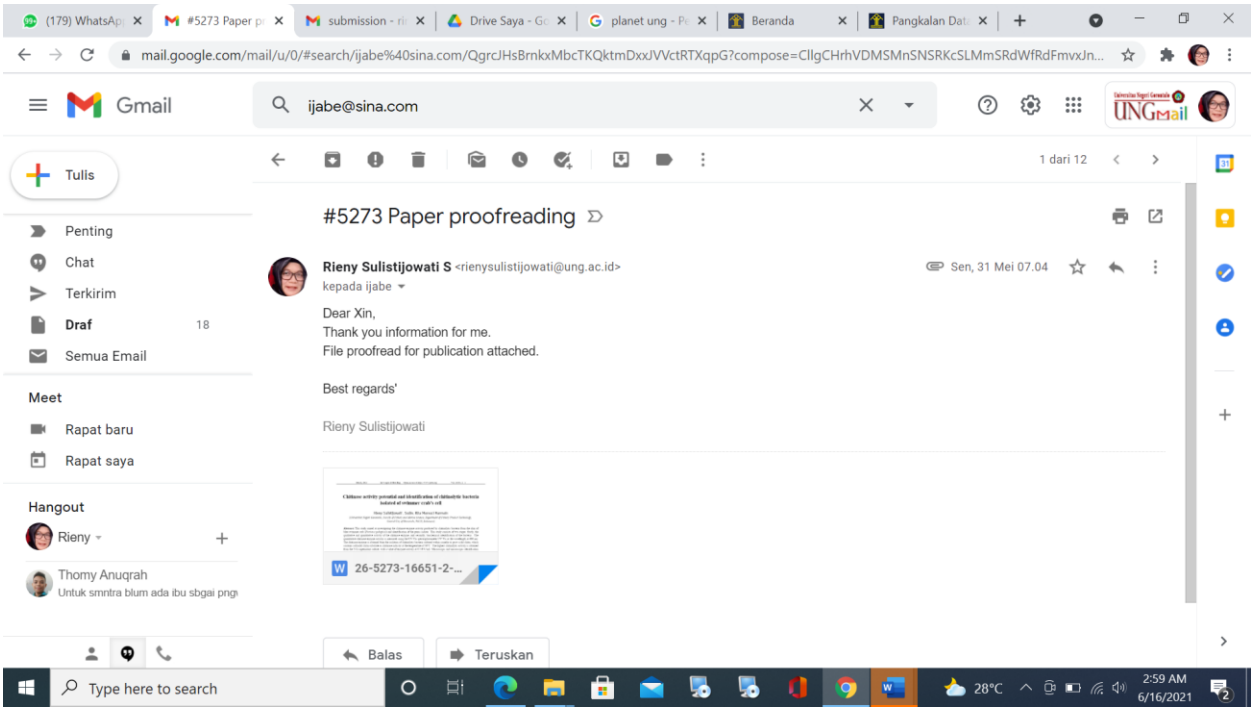
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Chitinase Activity Of Chitinolytic Bacteria from Blue Swimmer Crab's Cell (*Portunus pelagicus*)

Rieny Sulistijowati[1]*, Sudin[1], Rita Marsuci Harmain

[1]Universitas Negeri Gorontalo, Faculty of Fishery and Marine Science, Departement of Fishery Product Technology, Indonesia, Central City of Gorontalo, Sudirman Street No. 06 Postal Code 96128.

Corresponding author *rienysulistijowati@ung.ac.id

ABSTRACT

This study is aimed at investigating the chitinase enzyme activity produced by chitinolytic bacteria from the skin of blue swimmer crab (*Portunus pelagicus*) and identification of the genus isolate. This study consists of three stages; first, the screening of chitinolytic bacteria (isolation and macroscopic and microscopic identification); second, the qualitative and quantitative activity of the chitinase enzyme; third, biochemical identification of the bacteria. The quantitative chitinase enzyme activity is measured using spectrophotometer UV-Vis in the wavelength at 660 nm. The chitinase enzyme is obtained from the isolation of chitinolytic bacteria cultured within a media to grow solid chitin, which contains colloidal chitin substrate as chitinase inductor at the temperature of 30^{o} C. The chitinolytic bacteria isolate produces the highest chitinolytic index of 1. The highest chitinolytic activity is obtained from the 24 hours supernatant culture, with a value of enzyme activity at 0.149 U/mL Macroscopic and microscopic identification shows that the chitinolytic bacteria isolated are Gram-negative bacteria in the form of stem, whereas the biochemical cell shows the characteristics of the genus *Pseudomonas.*

Keywords: Protease, Biodegradable, Chitinolytic index, Identification, *Pseudomonas*.

I. INTRODUCTION

The implementation of biotechnology toward chitin, which keeps progressing, is the utilization of enzymes from microorganisms for biodegradation. In biodegradation, an

enzyme derived from microorganisms breaks large molecule or chitin polymer into utilizable products. In general, types of an enzyme that degraded the chitin are chitinase enzyme (Purkan, et al., 2016).

Microorganisms that degraded chitin, in general, are those derived from bacteria group. Chitinase enzyme produced by chitinolytic bacteria has the potential to degrade chitin due to the existence of the chitinase enzyme, which enables the conversion of abundantly available chitin into usable products. The bacteria that produce chitinase enzyme or chitinolytic bacteria can be found within the habitat that contains a high level of chitin, such as in the cell of the blue swimmer crab. Blue swimmer crab's cell (Portunus Pelagicus) can be obtained from the processing waste or fresh. Chitinase enzyme application can be informed of enzymatic production of chitin. The chitin can be produced enzymatically and chemically. The enzymatic method uses enzymes or bacteria for deproteinization by adding enzyme or by the involvement of chitinase to degrade chitin. Meanwhile, the chemical process is through demineralization by adding an acid or strong acids, such as HCl and NaOH (Younes et al., 2012).

Chemical synthesis of chitin is an easy but less environmentally friendly method, as it uses plenty of chemical ingredients (HCl and NaOH) (Soeka & Triana, 2016). Thus, an enzymatic method is considered better as it is easy, simple, fast, and without any chemical solvent, which hurts harms the environment, and minimizing the danger about to with concerning the utilization of chemical stuff. The downside of this method for industrial usage is that this method is quite expensive and needs an optimum condition for the enzyme to work maximally, and it is easy to be influenced by the environment (Chasanah, et al., 2014).

The needs for the chitin derivatives are increasing. Thus, research on chitinase enzyme activity through the isolation of bacteria from the blue swimmer crab is needed. A study by Arbia et al. (2013), which isolate chitinolytic bacteria to produce several bacteria, one of them is *Pseudomonas aeruginosa* bacteria isolated from crab's cell. Purkan (2016) production of chitinase enzyme from *Aspergillus niger* utilizing the blue swimmer crab's waste as inducer. Oh et al (2000) Protease produced by *Pseudomonas aeruginosa* K-187, the highest protease activity was as high as 21.2 U/ml, 10-fold that (2.2 U/ml) obtained before optimization. In common with all enzymes, external factors such as temperature, pH and type of media are important for the activity, catalytic efficiency, stability and proper functioning of proteases (Homaei et al, 2016). Chitinase activity of isolates chitinolytic bacteria can degrade different chitins.

This is due to the type of bacteria, growth pattern and enzyme activity need to be known to have a good degrading ability. Therefore, this study is aimed at identification chitinase producer bacteria of blue swimmer crab and tests the produced chitinase activity.

II. RESEARCH METHOD

Data obtained from this study are quantitative and qualitative data presented in the form of tables and figures.

Station and Laboratory

Fresh blue swimmer crab's cells are obtained from the crabs' farmer in Katialada village of Kwandang sub-district of North Gorontalo Regency, Gorontalo Province of

Indonesia. The isolation, identification, gram coloring and biochemical test of the chitinolytic bacteria are carried out at the Fish Quarantine Station Laboratory Quality Control and Fisheries Product Security Class I Gorontalo Province of Indonesia, and the chitinolytic bacteria enzyme activity test are carried out at the Pharmaceutical Laboratory of Universitas Negeri Gorontalo.

Material and Methods

The tools used in this study were test tube, Ose needle, vortex, measuring cup, Beaker glass, Erlenmeyer, centrifuge, centrifuge tube, *shaker*, pH meter, micropipette, micro tip, stirrer, magnetic stabilizer, petri dish, oven, crooked spoon, bunsen lamp, incubator, laminar air, analytical scales, Uv-vis spectrophotometer, and microscope.

Materials used in this study were blue swimmer crab's cell, chitin, chitin colloidal (chitin, condensed HCl, NaOH, aquadest), chitin agar (chitin colloidal, KH_2PO_4, $MgSO_4.7H_2O$, yeast extract, agar, aquadest), Luria broth (yeast extract, tripton, NaCl, aquadest), nutrient agar, aluminum foil, alcohol, crystal violet, iodine solution, safranin, glucose, sucrose, lactose, maltose, mannitol, triple sugar iron agar, motility indole ornitin, oxidative/fermentative, methyl red-Voges Proskauer, methyl-red, and sterile liquid paraffin.

Preparation of Blue Swimmer Crab's Cell Sample (P.pelagicus)

The sample fresh blue swimmer crab's cells are stored in a cool box with ice cubes layered with sealed and sterilized plastics. This is in for the sample to not have direct contact with the ice blocks.

Preparation of Colloidal Chitin

The preparation of colloidal chitin uses a partial hydrolysis method. According to Arnold and Solomon (1986), colloidal chitin is produced from dissolving 10 grams of chitin into 200 mL of HCL into 200 mL of concentrated HCl into a beaker glass; then the solution is settled for a night in a closed glass in the temperature of 4 ^{0}C. This solution is filtered using *glass wool*, then the filtrate is mixed with 100 mL of cool aquadest and added with NaOH 12 N to reach pH 7. The solution then centrifuged at the speed of 8,000 rpm for 20 minutes. The supernatant then disposed of, the sediment then added with aquadest, then centrifuged again using the 8,000 rpm speed for 20 minutes. The formed sediment is ready to use colloidal chitin.

Isolation of Chitinolytic Bacteria Produced from Blue Swimmer Crab's Cell (Portunus pelagicus)

Inoculation is carried out on media that contains colloidal chitin in the dilution of 10^{-1} to 10^{-6}. The isolation process of the bacteria is carried out using selective, isolated media called chitin agar media. The process of chitin agar media creation is by dissolving 2.5 grams of colloidal chitin into 5 mL of aquadest. After that 0.5 grams of $MgSO_4.7H_2O$; 0,1 g KH_2PO_4; 0.5 grams of yeast extract; 7.5 grams of agar and 495 mL of aquadest are added. All the mixed ingredients then boiled. The media is sterilized in the temperature of 121 ºC for 15-20 minutes. Five mL of sterilized media then pour into each petri dish. The last three dilution concentrates (10^{-4}, 10^{-5}, 10^{-6}) are inoculated into chitin agar media by

1 mL using a micropipette; then each petri dish is wrapped and incubated in the temperature of 30 ^{0}C for 48 hours.

Screening of Chitinolytic Bacteria

The purification of chitinolytic bacteria uses the strike plate method. The result from the isolation of these bacteria then goes through the purification of culture bacteria to obtain pure colony isolate. The first step in the purification of bacteria is selecting different bacteria colonies from the isolation process. The bacteria colony then inoculated in a surface of the chitin agar medium by using a sterilized does needle through the strike plate method to obtain a separated colony. The result from this process then incubated at a temperature of 30 ^{0}C for 48 hours (Kamil *et al.*, 2007). The next processes are macroscopic and microscopic observations.

Macroscopic identification is directly carried out, whereas microscopic identification is carried out through gram coloring. The gram coloring is carried out by taking one dose of isolated bacteria which diluted into 3 mL of sterilized aquadest, then 10 µl are taken and put into glass object and fixated. After that one drop of crystal violet (Gram A) is added into the solution for 1 minute then washed using flowing water and air-dried. When it is dried, one gram of iodine (Gram B) is added, then washed with flowing water. Further, the bacteria isolate is added with a 95% acetone alcohol solution (Gram C) for 30 seconds and washed with flowing water. After that, the isolate bacteria are added with Safranin (Gram D) for 2 minutes and washed using flowing water and air-dried. The isolate then observed under the microscope. Gram-positive bacteria will form purple color whereas Gram-negative bacteria will form red color (Cappuccino and Sherman, 2005).

Qualitative Activity of Chitinolytic Bacteria

Chitinase activity test of chitinolytic bacteria is carried out by putting a drop in chitin agar media. This test is to see the clear zone produced. In this test, one dose of the pure isolate is taken and inserted into the petri dish, which contains solidified chitin agar media. The bacteria are incubated at a temperature of 30 ^{0}C for 72 hours. The established clear area is observed and measured using the ruler (Chasanah, 2009).

The clear zone formed in the medium is the response toward the colloidal chitin added into the medium. From all pure isolates tested of their chitinolytic activity, one isolate that produces the largest chitinolytic index is taken.

Quantitative Activity of Chitinase Enzyme Test

One dose of inoculum is added into 100 mL of production medium (similar composition to a solid medium, but without agar) then incubated in the temperature of 30 ^{0}C and centrifuged in the speed of 170 rpm. Every three hours, 2 mL of cell culture is sampled for 33 hours. Then centrifuged in the temperature of 4 ºC using 10.000 rpm speed for 10 minutes, the formed supernatant is the raw extract of chitinase enzyme. The absorbance is then measured using Spectrophotometer UV/Vis in λ 660 nm (Purkan et al, 2014), the sample is carried out twice repetitions.

Biochemical Test

The biochemical test is carried out to identify and classify bacteria into their group of taxonomy. The principle of this biochemical test is if the bacteria are cultured in several

media, the bacteria will show macroscopic differences in their growth (Cappuccino and Sherman, 2005; Adyta *et al.*,2017) were; Carbohydrate fermentation test to find out the bacteria's ability in fermenting carbohydrate by preparing the carbohydrate broth which consists of glucose, sucrose, maltose, and mannitol; MR-VP (methyl red-Voges Proskauer) test to inoculate bacteria into a medium, which incubated in the temperature of 30 ºC for 24 hours by adding methyl red reagent and KOH, to observe the bacteria ability in producing the mix acid and acetylenes; Citrate test by inoculating bacteria into a medium and incubated in the temperature of 30 ºC for 24 hours by adding BTB (bromothymol blue) reagent, then observe the ability of the bacteria to use citrate as the only source of carbon; H_2S (Hydrogen Sulfide) test to inoculate the bacteria into SIM (sulfide indole motility) which incubated for 24-48 hours in the temperature of 30 ºC, then observe the ability of the bacteria to produce H_2S which signify by the existence of black sediment; O/F (oxidation/fermentation) test by inoculating the bacteria into the O/F medium, which incubated for 24 hours in the temperature of 30 ºC, then observe the ability of the bacteria to use carbohydrate through fermentation or oxidation; TSIA (triple sugar iron agar) test by inoculating bacteria into TSIA media, which incubated for 24-48 hours in the temperature of 30ºC, then observe the ability of the bacteria in fermenting glucose, lactose, and sucrose; Indole test by inoculating bacteria into indole media, which incubated for 24 hours in the temperature of 30 ºC, then observe the ability of bacteria in degrading the tryptophan amino acid in the medium.

III. RESULTS AND DISCUSSION

Isolation Result and Purification of Chitinolytic Bacteria

The isolation result following 48 hours incubation in the temperature of 30 ^{0}C produces bacteria that grow in the chitin agar media. It is found that bacteria only grow in Petri dishes 10^{-4}. These bacteria then separated based on the appearance of its colony, different shape, and color. The result is classified into three different colonies, white, light brown, and yellow colored.

The result obtained from the purification process following 48 hours incubation in the temperature of 30 ^{0}C shows a clear zone that formed in the colony of the bacteria. However, clear zones are only found in white and light brown-colored bacteria. Further, these two isolates are macroscopically and microscopically identified.

Macroscopic and Microscopic Identification Result

Based on the result of macroscopic and microscopic identification, it shows that chitinolytic bacteria shapes are round, white and light brown-colored, the edge of the colony are complete and have Gram-negative. The morphology of the colony and the cell of chitinolytic bacteria is are presented in Figure 1 and Table 1.

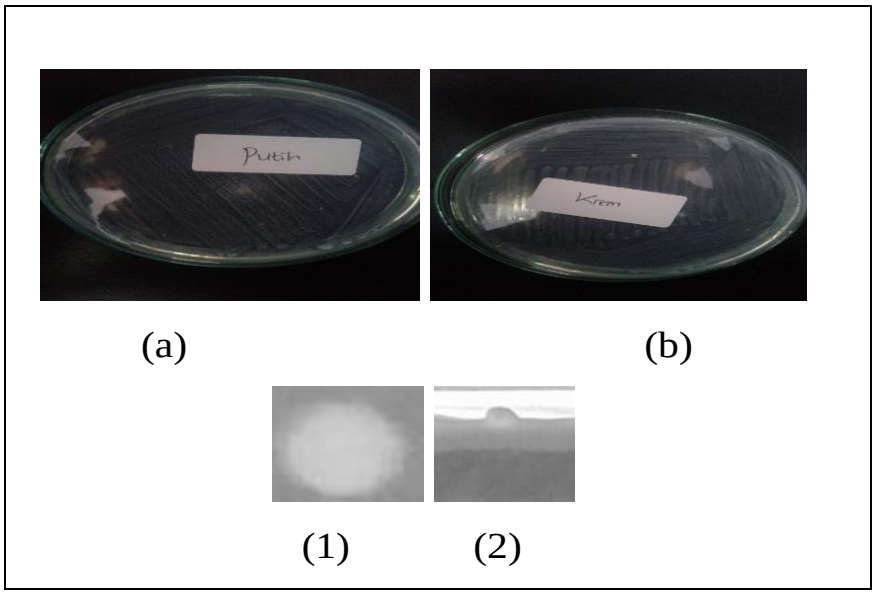

Figure 1. Chitinolytic Bacteria Isolate.
(a) Isolate R1 (white colony), (b) Isolate R2 (light brown colony),
(1) Shape of the Colony, (2) Colony Elevation.

Table 1. Characteristics of Isolates Chitinolytic Bacteria

No.	Isolates Bacteria	Colony color	Colony Forms	Colony Elevation	Gram Stain
1	R1	White	Circular	Convex	Rod (-)
2	R2	Light brown	Circular	Convex	Rod (-)

As seen in Table 1, the R1 isolate shows a white-colored colony, meanwhile, the R2 isolate shows a light brown-colored colony. Meanwhile, from the shape/form and elevation of the colony, there are no differences between isolate R1 and R2, both colonies have a circular shape and convex elevation. The staining/coloring result of the bacteria is shown in Figure 2.

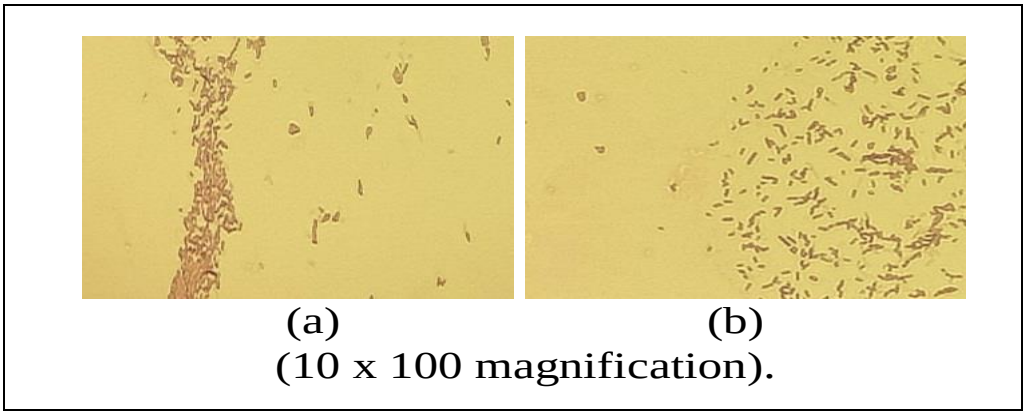

Figure 2. Gram staining/coloring of the Chitinolytic Bacteria
(a) Gram-Negative of the isolate R1, (b) Gram-negative of the isolate R2

The result of gram staining in these two chitinolytic bacteria isolates R1, and R2 shows the Gram-negative result. This is signified by changes of color of these two isolates into the red after the gram staining.

Cappuccino and Sherman (2005) argue that Gram-negative changes its color into the red due to the violet crystal color is dissolved during the administration of acetone solution, and takes the red color of the Safranine. In general, Gram-negative bacteria have a high lipid cell wall. Hence, the lipid is dissolved in the acetone solution.

Qualitative Screening of Chitinolytic Bacteria Activity

Enzyme activity of chitinolytic bacteria is shown by the appearance of a clear zone formed around the colony. This clear zone proves that the isolate can degrade the chitin substrate within the chitin agar media. Harman et al, (1993) argue that chitinolytic bacteria are competent bacteria to produce chitinase enzymes and utilize chitinase to assimilate chitin as a source of carbon and nitrogen. The enzyme activity of the chitinolytic bacteria is seen in the white and cream-colored of the isolates as seen in Figure 3.

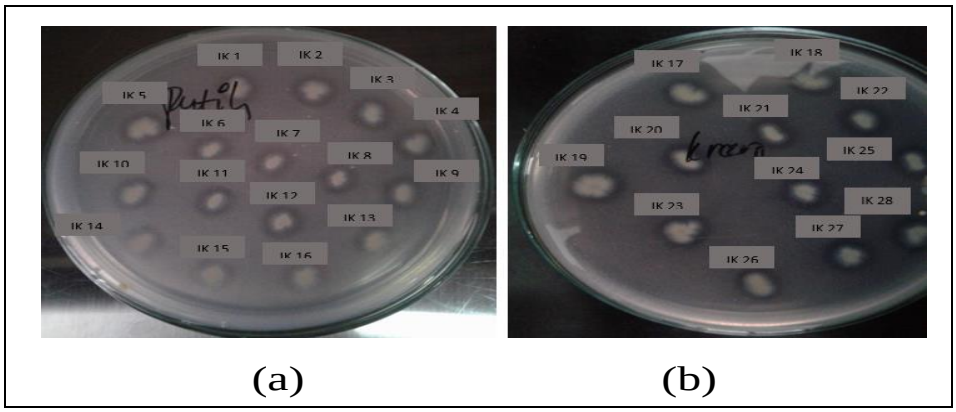

Figure 3. The clear zone formed in chitin agar by chitinolytic bacteria, which show different Chitinolytic Index (a) Isolate R1 (IK 1-16) (b) Isolate R2 (IK 17-28).

Bacteria isolates produced from the blue swimmer crab's cell show the existence of a clear zone. This clear zone is formed due to the chitinase enzyme activity released from the cells. Bacteria to degrade macromolecules of the chitin into smaller chitin molecules. Thus, bacteria can digest nutrition from these small molecules. This is due to the different levels of bacteria adaptation toward their environment. In a selective solid media, it is known that bacteria are potential as chitinase producer, which signify by the formation of a clear zone around the colony. The more enzymes produced, the wider the clear zone that will be formed as the number of degraded chitin is increasing, Harman et al, (1993).

Isolation and selection result of the bacteria cultured in chitin media points out that there are bacteria that grow with different chitinolytic index (Table 2).

Table 2. Chitinolytic Index of the Bacteria

Isolate	Type	Diameter Zone (mm)	Diameter colony (mm)	Index of chitinolytic
	IK 1	2	5	0.4
	IK 2	2	6	0.3
	IK 3	2	5	0.4
	IK 4	1.5	5	0.3
	IK 5	2	6.5	0.33
	IK 6	2	3	0,6
	IK 7	2	3.5	0.56
	IK 8	2.5	3	0.83
R1	IK 9	2	3.5	0.56
	IK 10	2	5	0.4
	IK 11	2.5	2.5	1
	IK 12	2.5	3.5	0.71
	IK 13	2	5	0.4
	IK 14	2	4	0.5
	IK 15	2.5	4.5	0.56
	IK 16	2	5	0.4
	IK 17	1,5	5.5	0.27
	IK 18	2	5	0.4
	IK 19	1.5	7	0.21
	IK 20	1.5	5.5	0.27
	IK 21	2	2.5	0.8
	IK 22	2	8	0.25
R2	IK 23	2	5.5	0.36
	IK 24	1,5	5	0.3
	IK 25	2	5.5	0.36
	IK 26	2	5.5	0.36
	IK 27	2	7	0.29
	IK 28	1.5	6.5	0.23

Note: Isolate R1 (Chitinolytic Index/CI 1-16), Isolate R2 (Chitinolytic Index/C17-18

The study shows that CI 11 of the R1 isolate has the largest Chitinolytic Index by 1. Chasanah (2009) who found that the largest chitinolytic index produced from the bacteria is 2.58 supports this result. The difference in this chitinolytic index is due to the different levels of bacteria adaptation toward its environment. Also, this difference is due to the

different types of bacteria. The chitinolytic index shows the ability of the microbes to degrade chitin. The more enzyme produced, the wider the clear zone produced as more chitin is degraded.

Quantitative Activity of the Chitinolytic Bacteria Enzyme

The activity enzyme of chitinolytic bacteria is presented in Figure 4.

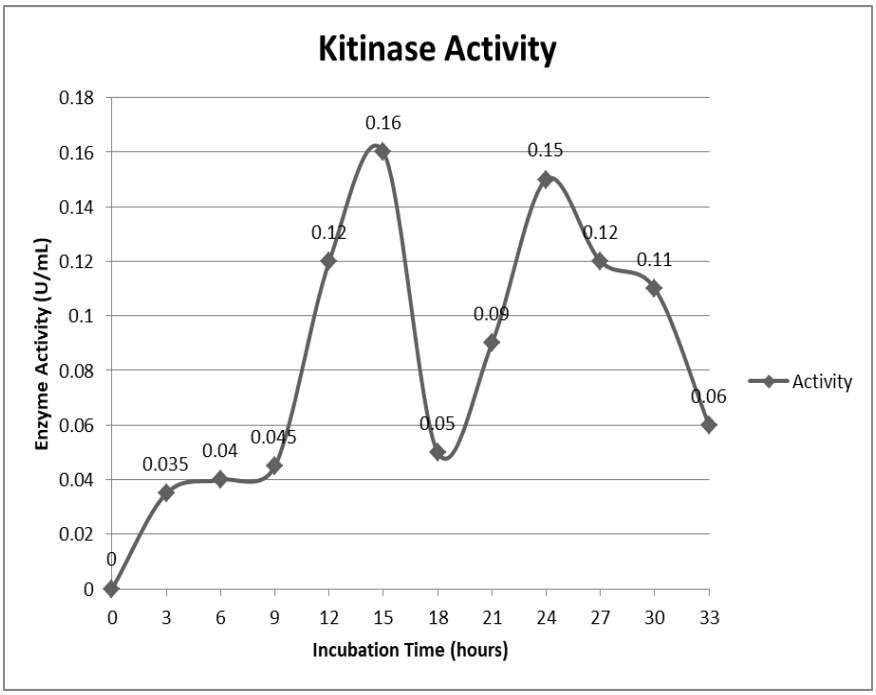

Figure 4. Chitinase Enzyme Activity Curve

The activity of the chitinolytic bacteria enzyme is essential to be known to find out the ability of the bacteria to produce the enzyme in 33 hours with an interval of 3 hours. There are several increasing and decreasing stages in chitinase activity. The first inclination happens during the incubation time of 0 hours to 12 hours. The second increase of the enzyme activity shows that the substrate is starting to be hydrolyzed to produce the chitinase enzyme. Hence, bacteria can digest nutrition. Patil et al, (2000) wrote that bacteria produce extracellular chitinase to take on nutrition. Following this inclination, there is the first declining phase on the 15th to the 18th hour of incubation. The decrease of this enzyme activity is due to other compounds (aside from N-Acetyl glucosamine) that triggers the decrease of enzyme production.

This phenomenon is due to the existence of other chitin-degrading enzymes produced by the bacteria. Fukamizo (2000) argues that colloidal chitin also can be hydrolyzed by deacetylating chitin produced by chitosan and chitosanase which produce chitobiose. Following this declining phase, the chitinase activity climbs up in the incubation time of a 21st hour to its highest chitinase activity that can be obtained from the supernatant culture in the incubation of time of a 24th hour, which stated by the value of enzyme activity by 0.149 U/mL. One unit of chitinase enzyme activity is defined as several enzymes needed to release one mmol NAG/minute. This result is different from the result of the enzymes

activity test carried out by Purkan et al (2014) who found that that the highest enzyme activity is in the 18th hour of incubation time, which started with the value of enzyme activity by 0.3850 U/mL. Also, Orinda et al (2015) argue that the ability of the bacteria to produce chitinase highly varied. Factors such as different types of bacteria, the growth rate of each isolate in the medium, or laboratory treatment during the experiment can be factors that influence variation in the produced enzyme activity.

The rebound of enzyme activity shows that there is more of the substrate being hydrolyzed. The chitinase enzyme activity is steadily increasing until it reaches optimum incubation time. Following the reach of this optimum incubation time, the enzyme activity decreases due to the accumulation of hydrolyzed products, which can further inhibit the enzyme activity. This is characterized by the decrease of enzyme activity on the incubation time of hour 27 to hour 33. Fukamizo (2000) argues that this decrease of chitinase enzyme activity after the optimum incubation time is due to the changes in the state of the enzyme ion and the state of substrate ion which caused denaturation of enzyme which followed by the loose of enzyme catalytic activity. Besides, there are also change in the tertiary structure of the enzyme due to denaturation, which made the hydrophobic amino acid group within the enzyme to come into contact with water, thus, the solubility of the enzyme weakens. The decrease of chitinase solubility causes a gradual decrease in enzyme activity.

Chitinolytic bacteria isolates show unstable chitinase activity (fluctuate). Orinda *et al.*, (2015) suggest that this may be due to the isolate that produces the chitinase at the beginning of its growth. In line with the utilization of nutrition for growth, it is also suspected that chitinase is also used by bacteria as a source of protein, thus its chitinase activity decreases.

The decrease of enzyme activity can also be caused by factors such as temperature, pH, substrate and biomass during treatment in the laboratory. The temperature has two main influences on the reaction and the denaturation. The influence of reaction toward the enzyme is that the increase of temperature will accelerate the reaction process, while the decrease in the temperature will cause the reaction to slow down. When the temperature reaches a certain limit, it will cause denaturation. Besides, when the pH of the environment is too acid or base, enzyme denaturation can also happen. Reaction speed catalyzed by the enzyme is highly influenced by substrate concentration. In the low level of substrate concentration, reaction speed by catalyzed by the enzyme can also be very low. In reverse, reaction speed will increase along with the increase of substrate concentrate up to certain points that is the maximum reaction speed limit. When this saturated point of the enzyme has been reached, it will not function properly. Lastly, the number of bacteria inoculum (biomass) inserted into the media also strongly influence the enzyme activity.

Biochemical Test

The biochemical test toward the characteristics of chitinolytic bacteria is carried out by fermenting bacteria in the various sources of nutrition. The biochemical test result of chitinolytic bacteria is presented in Table 3.

Table 3. Biochemical Characteristics of Chitinolytic

Bacteria Isolate R1

No	Test	Results
1	Glucose fermentation	Negative
2	Sucrose fermentation	Negative
3	Lactose fermentation	Negative
4	Maltose fermentation	Negative
5	Mannitol fermentation	Negative
6	Citrate Use	Negative
7	Sulfide Indole Motility	Negative
8	Triple Sugar Iron Agar	Alkaline/Alkaline
9	Methyl Red Reaction	Negative
10	Voges Proskauer Reaction	Negative
11	Indole production	Negative
12	Oxidase/Fermentative activity	Negative

Fermentation test in several types of carbohydrate (glucose, maltose, sucrose, mannitol, lactose) shows that all fermentation reaction is negative. This is characterized by the unchanging red color of the carbohydrate media. Aditi et al, (2017) argue that when the color of the medium in the carbohydrate test turns into yellow, it means that the colony forms acid from that carbohydrate.

A citrate test is carried out to find out the ability of the chitinolytic bacteria isolates to utilize citrate as the only source of carbon and energy. When a microorganism can use citrate, there will be an increase of pH and change in the color of the media into a blue color. In this study, the citrate test reveals that the chitinolytic bacteria cannot utilize citrate as the only source of carbon. This is shown by the unchanging green color of the media, which means that the test result is negative. Hemraj et al, (2013) argue that positive test results in the citrate test are shown when the color changes from green to blue.

Further, the H_2S test result in SIM is negative. This negative result is reached when microorganism has no ability to hydrolyzed heavy metal within the media. H_2S is produced by several types of microorganisms, which can break or degrade amino acid within the sulfur (S). The existence of H_2S can be observed by adding several crystals of heavy metals into the media.

The reaction observable in the TSIA test shows a red color, which means that there is no change of color in both vertical and slight agar. This indicates that the bacteria are unable to ferment sugar. Amano et al, (2014) opine that in the vertical agar if the bacteria can ferment glucose, the color of the media will change from red to yellow. Whereas in slight agar, if the bacteria can ferment lactose and sucrose, the color of the media will change into yellow, meanwhile, when there is no fermentation process of lactose and sucrose, the color will not change.

Methyl red test also reveals a negative result. This is shown by the unchanging color of the media which does not change into yellow even after the addition of methyl red reagent. Hemraj et al, (2013) wrote that the red color signifies the positive test result, and if

the color of the broth is yellow, then the result of the test is negative. Similarly, the Voges Proskauer test also shows a negative result. This is evident after the addition of the KOH solution; the color does not change. The VP test will be stated as positive when there is a form of acid, which signifies by the changes of medium color into pink after the KOH solution is added. Meanwhile, the indole test also shows a negative result. This result is obtained after the *reagent* Kovac is added, which signify by the formation of a yellow ring. The existence of indole is detected by Kovac reagent and the formation of a red ring.

The objective of the oxidizing fermentative test is to find out the oxidation and fermentation characteristics of bacteria toward glucose. Based on the result of the study on the O/F test, it does not show either oxidation or fermentation. This is evident when the media either without liquid paraffin or without paraffin at all do not change color. Bacteria are said to be fermentative when both inoculated media change color into yellow. Cowan and Steels (2003) state that bacteria are oxidative when tube sealed with no paraffin changes color into yellow and the tube sealed with paraffin does not change color.

As written (Cappuccino and Sherman, 2005) the morphology test result (macroscopic and microscopic tests) of chitinolytic bacteria have bar cell and Gram-negative. The biochemical test of the chitinolytic bacteria consists of carbohydrate, citrate, sulfide indole motility, triple sugar iron agar, MR-VP, Indole and O/F test should obtain a negative results for as indicators of a genus of *Pseudomonas*. Several studies to determine chitinolytic bacteria from the genus of *Pseudomonas* are studies carried by Purkan et al, (2016) that utilized blue swimmer crab's waste as an inducer to the production of chitinase enzyme from *Aspergillus niger*. A study by Arbia *et al.* (2013), which isolate chitinolytic bacteria to produce several bacteria, one of them is *Pseudomonas aeruginosa* bacteria isolated from crab's cell. Genus *Pseudomonas* generally has bar cell shape and Gram-negative.

CONCLUSION

This study concludes that chitinolytic bacteria isolated from blue swimmer crab's cell (*Portunus pelagicus*) genus of Pseudomonas. This is proven by the formation of the clear zone with a chitinolytic index of 1. The characteristics of chitinolytic bacteria from this blue swimmer crab show that the bacteria are a bar shape and Gram-negative bacteria. The highest chitinase activity is obtained from the supernatant culture obtained in the 24th hour, in which enzyme activity value is 0.149 U/mL as a good potential to degradable chitin ability.

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Chitinase Activity Potential And Identification Of Chitinolytic Bacteria Isolated Of Swimmer Crab's Cell

Rieny Sulistijowati[1]*, Sudin[1], Rita Marsuci Harmain[1]

[1]Universitas Negeri Gorontalo, Faculty of Fishery and Marine Science, Departement of Fishery Product Technology, Indonesia, Central City of Gorontalo, Sudirman Street No. 06 Postal Code 96128.

Corresponding author *rienysulistijowati@ung.ac.id

ABSTRACT

This study is aimed at investigating the chitinase enzyme activity produced by chitinolytic bacteria from the skin of blue swimmer crab (*Portunus pelagicus*) and identification of the genus isolate. This study consists of two stages: firstly, qualitative and quantitative activity of the chitinase enzyme; and secondly, biochemical identification of the bacteria. The quantitative chitinase enzyme activity is measured using the UV-Vis spectrophotometer UV-Vis at the wavelength at 660 nm. The chitinase enzyme is obtained from the isolation of chitinolytic bacteria cultured within a media to grow solid chitin, which contains colloidal chitin substrate as chitinase inductor at the temperature of 30º C. The highest chitinolytic activity is obtained from the 24 hours supernatant culture, with a value of enzyme activity at 0.149 U/mL. Macroscopic and microscopic identification shows that the chitinolytic bacteria isolate R1, whereas the biochemical cell shows the characteristics of the genus *Pseudomonas.*

Keywords: Biodegradable, Chitinase, Spectrophotometer, *Portunus pelagicus*, *Pseudomonas*.

I. INTRODUCTION

The on earth chitin is among the most abundant biomass present. Chitinase plays an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. The implementation of biotechnology toward chitin, which keeps progressing, is the utilization of enzymes from microorganisms for biodegradation. In biodegradation, an enzyme derived from microorganisms breaks large molecule or chitin polymer into utilizable products. In general, types of an enzyme that degraded the chitin are chitinase enzyme (Bhattacharya, et al., 2008).

Microorganisms that degraded chitin, in general, are those derived from bacteria group. Chitinase enzyme produced by chitinolytic bacteria has the potential to degrade chitin due to the existence of the chitinase enzyme, which enables the conversion of abundantly available chitin into usable products. The bacteria that produce chitinase enzyme or chitinolytic bacteria can be found within the habitat that contains a high level of chitin, such as in the cell of the blue swimmer crab. Blue swimmer crab's cell (*Portunus pelagicus*) can be obtained from the processing waste or fresh. Chitinase enzyme application can be informed of enzymatic production of chitin. The chitin can be produced enzymatically and chemically. The enzymatic method uses enzymes or bacteria for deproteinization by adding enzyme or by the involvement of chitinase to degrade chitin. Meanwhile, the chemical process is through demineralization by adding an acid or strong acids, such as HCl and NaOH (Younes et al., 2012).

A study by Arbia et al. (2013), which isolate chitinolytic bacteria to produce several bacteria, one of them is *Pseudomonas aeruginosa* bacteria isolated from crab's cell. Purkan (2016) production of chitinase enzyme from *Aspergillus niger* utilizing the blue swimmer crab's waste as inducer. Oh et al (2000) Protease produced by *Pseudomonas aeruginosa* K-187, the highest protease activity was as high as 21.2 U/ml, 10-fold that (2.2 U/ml) obtained before optimization. In common with all enzymes, external factors such as temperature, pH and type of media are important for the activity, catalytic efficiency, stability and proper functioning of proteases (Homaei et al, 2016). Chitinase activity of isolates chitinolytic bacteria can degrade different chitins.

The needs for the chitin derivatives are increasing. Thus, research on chitinase enzyme activity through the isolation of bacteria from the blue swimmer crab is needed. Observe primerly that two isolates chitinolitic bacteria from the skin of blue swimmer crab in Katialada village of Kwandang sub-district of North Gorontalo Regency, Gorontalo Province of Indonesia. Sudin et al. (2020), the result obtained from the purification process following 48 hours incubation in the temperature of 30 ^{0}C shows a clear zone that formed in the colony of the bacteria. However, clear zones are only found in white and light brown-colored bacteria. Further, these two isolates are macroscopically and microscopically identified. The R1 isolate shows a white-colored colony, meanwhile, the R2 isolate shows a light brown-colored colony. Meanwhile, from the shape/form and elevation of the colony, there are no differences between isolate R1 and R2, both colonies have a circular shape and convex elevation. The result of gram staining in these two chitinolytic bacteria isolates R1, and R2 shows the Gram-negative result. This is signified by changes of color of these two isolates into the red after the gram staining. The study shows that CI 11 of the R1 isolate has the largest Chitinolytic Index by 1, the chitinolytic index shows the ability of the microbes to degrade chitin. The more enzyme produced, the wider the clear zone produced as more chitin is degraded.

This is due to the type of bacteria, growth pattern and enzyme activity need to be known to have a good degrading ability. Therefore, this study is aimed at tests the produced chitinase activity and identification chitinase producer bacteria of blue swimmer crab.

II. RESEARCH METHOD

Data obtained from this study are quantitative and qualitative data presented in the form of tables and figures.

Station and Laboratory

Isolate R1 obtain from fresh blue swimmer crab's cells are obtained from the crabs' farmer in Katialada village of Kwandang sub-district of North Gorontalo Regency, Gorontalo Province of Indonesia. The identification biochemical test of the chitinolytic bacteria are carried out at the Fish Quarantine Station Laboratory Quality Control and Fisheries Product Security Class I Gorontalo Province of Indonesia, and the chitinolytic bacteria enzyme activity test are carried out at the Pharmaceutical Laboratory of Universitas Negeri Gorontalo.

Material and Methods

The tools used in this study were test tube, Ose needle, vortex, measuring cup, Beaker glass, Erlenmeyer, centrifuge, centrifuge tube, *shaker*, pH meter, micropipette, micro tip, stirrer, magnetic stabilizer, petri dish, oven, crooked spoon, bunsen lamp, incubator, laminar air, analytical scales, Uv-vis spectrophotometer, and microscope.

Materials used in this study were R1 isolated of blue swimmer crab's cell, chitin, chitin colloidal (chitin, condensed HCl, NaOH, aquadest), chitin agar (chitin colloidal, KH_2PO_4, $MgSO_4.7H_2O$, yeast extract, agar, aquadest), Luria broth (yeast extract, tripton, NaCl, aquadest), nutrient agar, aluminum foil, alcohol, crystal violet, iodine solution, glucose, sucrose, lactose, maltose, mannitol, triple sugar iron agar, motility indole ornitin, oxidative/fermentative, methyl red-Voges Proskauer, methyl-red, and sterile liquid paraffin.

Quantitative Activity of Chitinase Enzyme Test

One dose of inoculum is added into 100 mL of production medium (similar composition to a solid medium, but without agar) then incubated in the temperature of 30 ^{0}C and centrifuged in the speed of 170 rpm. Every three hours, 2 mL of cell culture is sampled for 33 hours. Then centrifuged in the temperature of 4 ºC using 10.000 rpm speed for 10 minutes, the formed supernatant is the raw extract of chitinase enzyme. The absorbance is then measured using Spectrophotometer UV/Vis in λ 660 nm (Purkan et al, 2014), the sample is carried out twice repetitions.

Biochemical Test

The biochemical test is carried out to identify and classify bacteria into their group of taxonomy. The principle of this biochemical test is if the bacteria are cultured in several media, the bacteria will show macroscopic differences in their growth (Cappuccino and Sherman, 2005; Adyta *et al.*,2017) were; Carbohydrate fermentation test to find out the bacteria's ability in fermenting carbohydrate by preparing the carbohydrate broth which consists of glucose, sucrose, maltose, and mannitol; MR-VP (methyl red-Voges Proskauer) test to inoculate bacteria into a medium, which incubated in the temperature of 30 ºC for 24 hours by adding methyl red reagent and KOH, to observe the bacteria ability in producing the mix acid and acetylenes; Citrate test by inoculating bacteria into a medium and incubated in the temperature of 30 ºC for 24 hours by adding BTB (bromothymol blue) reagent, then observe the ability of the bacteria to use citrate as the only source of carbon; H_2S (Hydrogen Sulfide) test to inoculate the bacteria into SIM (sulfide indole motility) which incubated for 24-48 hours in the temperature of 30 ºC, then observe the ability of the bacteria to produce H_2S which signify by the existence of black sediment; O/F (oxidation/fermentation) test by inoculating the bacteria into the O/F medium, which incubated for 24 hours in the temperature of 30 ºC, then observe the ability of the bacteria to use carbohydrate through fermentation or oxidation; TSIA (triple sugar iron agar) test by inoculating bacteria into TSIA media, which incubated for 24-48 hours in the temperature of 30ºC, then observe the ability of the bacteria in fermenting glucose, lactose, and sucrose; Indole test by inoculating bacteria into indole media, which incubated for 24 hours in the temperature of 30 ºC, then observe the ability of bacteria in degrading the tryptophan amino acid in the medium.

III. RESULTS AND DISCUSSION

Quantitative Activity of the Chitinolytic Bacteria Enzyme

Data and standard deviation presented in Table 1.

Replication	Incubation (hours)											
	0	3	6	9	12	15	18	21	24	27	30	33
1	0	0.04	0.05	0.04	0.13	0.15	0.06	0.08	0.14	0.115	0.1	0.09
2	0	0.025	0.03	0.05	0.14	0.17	0.08	0.1	0.16	0.135	0.12	0.07
Mean	0	0.0325	0.04	0.045	0.135	0.16	0.07	0.09	0.15	0.125	0.11	0.08
Standard deviation	0	0.01061	0.01414	0.00707	0.0070711	0.01414	0.01414	0.01414	0.01414	0.01414	0.01414	0.01414

The activity enzyme of chitinolytic bacteria is presented in Figure 1.

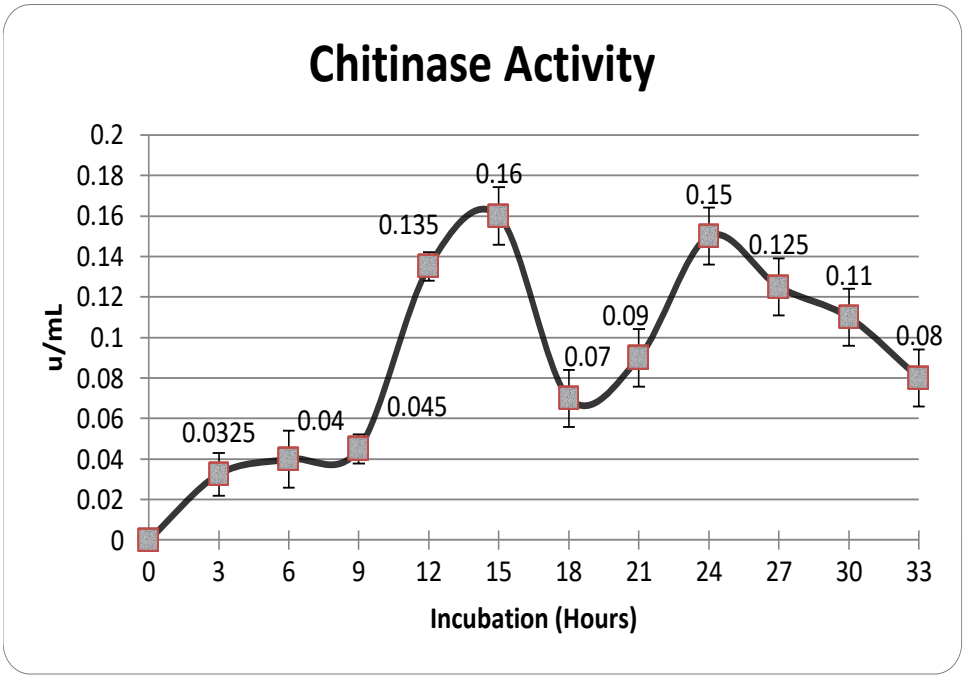

Figure 1. Chitinase Enzyme Activity Curve

The activity of the chitinolytic bacteria enzyme is essential to be known to find out the ability of the bacteria to produce the enzyme in 33 hours with an interval of 3 hours. There are several increasing and decreasing stages in chitinase activity. The first inclination happens during the incubation time of 0 hours to 12 hours. The second increase of the enzyme activity shows that the substrate is starting to be hydrolyzed to produce the chitinase enzyme. Hence, bacteria can digest nutrition. Patil et al, (2000) wrote that bacteria produce extracellular chitinase to take on nutrition. Following this inclination, there is the first declining phase on the 15th to the 18th hour of incubation. The decrease of

this enzyme activity is due to other compounds (aside from N-Acetyl glucosamine) that triggers the decrease of enzyme production.

This phenomenon is due to the existence of other chitin-degrading enzymes produced by the bacteria. Fukamizo (2000) argues that colloidal chitin also can be hydrolyzed by deacetylating chitin produced by chitosan and chitosanase which produce chitobiose. Following this declining phase, the chitinase activity climbs up in the incubation time of a 21st hour to its highest chitinase activity that can be obtained from the supernatant culture in the incubation of time of a 24th hour, which stated by the value of enzyme activity by 0.149 U/mL. One unit of chitinase enzyme activity is defined as several enzymes needed to release one mmol NAG/minute. This result is different from the result of the enzymes activity test carried out by Purkan et al (2014) who found that that the highest enzyme activity is in the 18th hour of incubation time, which started with the value of enzyme activity by 0.3850 U/mL. Also, Orinda et al (2015) argue that the ability of the bacteria to produce chitinase highly varied. Factors such as different types of bacteria, the growth rate of each isolate in the medium, or laboratory treatment during the experiment can be factors that influence variation in the produced enzyme activity.

The rebound of enzyme activity shows that there is more of the substrate being hydrolyzed. The chitinase enzyme activity is steadily increasing until it reaches optimum incubation time. Following the reach of this optimum incubation time, the enzyme activity decreases due to the accumulation of hydrolyzed products, which can further inhibit the enzyme activity. This is characterized by the decrease of enzyme activity on the incubation time of hour 27 to hour 33. Fukamizo (2000) argues that this decrease of chitinase enzyme activity after the optimum incubation time is due to the changes in the state of the enzyme ion and the state of substrate ion which caused denaturation of enzyme which followed by the loose of enzyme catalytic activity. Besides, there are also change in the tertiary structure of the enzyme due to denaturation, which made the hydrophobic amino acid group within the enzyme to come into contact with water, thus, the solubility of the enzyme weakens. The decrease of chitinase solubility causes a gradual decrease in enzyme activity.

Chitinolytic bacteria isolates show unstable chitinase activity (fluctuate). Orinda *et al.*, (2015) suggest that this may be due to the isolate that produces the chitinase at the beginning of its growth. In line with the utilization of nutrition for growth, it is also suspected that chitinase is also used by bacteria as a source of protein, thus its chitinase activity decreases.

The decrease of enzyme activity can also be caused by factors such as temperature, pH, substrate and biomass during treatment in the laboratory. The temperature has two main influences on the reaction and the denaturation. The influence of reaction toward the enzyme is that the increase of temperature will accelerate the reaction process, while the decrease in the temperature will cause the reaction to slow down. When the temperature reaches a certain limit, it will cause denaturation. Besides, when the pH of the environment is too acid or base, enzyme denaturation can also happen. Reaction speed catalyzed by the enzyme is highly influenced by substrate concentration. In the low level of substrate concentration, reaction speed by catalyzed by the enzyme can also be very low. In reverse, reaction speed will increase along with the increase of substrate concentrate up to certain points that is the maximum reaction speed limit. When this saturated point of the enzyme

has been reached, it will not function properly. Lastly, the number of bacteria inoculum (biomass) inserted into the media also strongly influence the enzyme activity.

Biochemical Test

Chitinolytic bacteria R1 isolated of Swimmer Crab's Cell presented in Figure 2.

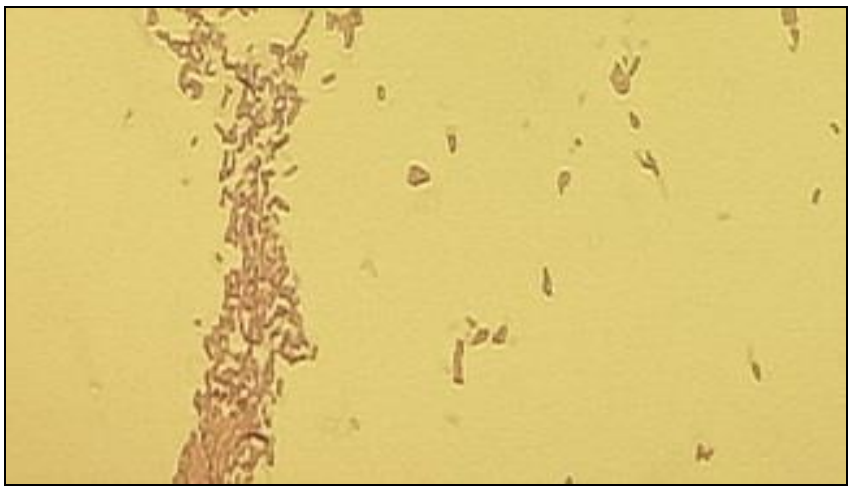

Figure 2. Isolate R1 (10x100 magnification)

The biochemical test toward the characteristics of chitinolytic bacteria is carried out by fermenting bacteria in the various sources of nutrition. The biochemical test result of chitinolytic bacteria is presented in Table 1.

Fermentation test in several types of carbohydrate (glucose, maltose, sucrose, mannitol, lactose) shows that all fermentation reaction is negative. This is characterized by the unchanging red color of the carbohydrate media. Aditi et al, (2017) argue that when the color of the medium in the carbohydrate test turns into yellow, it means that the colony forms acid from that carbohydrate.

A citrate test is carried out to find out the ability of the chitinolytic bacteria isolates to utilize citrate as the only source of carbon and energy. When a microorganism can use citrate, there will be an increase of pH and change in the color of the media into a blue color. In this study, the citrate test reveals that the chitinolytic bacteria cannot utilize citrate as the only source of carbon. This is shown by the unchanging green color of the media, which means that the test result is negative. Hemraj et al, (2013) argue that positive test results in the citrate test are shown when the color changes from green to blue.

Table 2. Biochemical Characteristics of Chitinolytic Bacteria Isolate R1

No	Test	Results
1	Glucose fermentation	Negative
2	Sucrose fermentation	Negative
3	Lactose fermentation	Negative
4	Maltose fermentation	Negative
5	Mannitol fermentation	Negative
6	Citrate Use	Negative
7	Sulfide Indole Motility	Negative
8	Triple Sugar Iron Agar	Alkaline/Alkaline
9	Methyl Red Reaction	Negative
10	Voges Proskauer Reaction	Negative
11	Indole production	Negative
12	Oxidase/Fermentative activity	Negative

Further, the H_2S test result in SIM is negative. This negative result is reached when microorganism has no ability to hydrolyzed heavy metal within the media. H_2S is produced by several types of microorganisms, which can break or degrade amino acid within the sulfur (S). The existence of H_2S can be observed by adding several crystals of heavy metals into the media.

The reaction observable in the TSIA test shows a red color, which means that there is no change of color in both vertical and slight agar. This indicates that the bacteria are unable to ferment sugar. Amano et al, (2014) opine that in the vertical agar if the bacteria can ferment glucose, the color of the media will change from red to yellow. Whereas in slight agar, if the bacteria can ferment lactose and sucrose, the color of the media will change into yellow, meanwhile, when there is no fermentation process of lactose and sucrose, the color will not change.

Methyl red test also reveals a negative result. This is shown by the unchanging color of the media which does not change into yellow even after the addition of methyl red reagent. Hemraj et al, (2013) wrote that the red color signifies the positive test result, and if the color of the broth is yellow, then the result of the test is negative. Similarly, the Voges Proskauer test also shows a negative result. This is evident after the addition of the KOH solution; the color does not change. The VP test will be stated as positive when there is a form of acid, which signifies by the changes of medium color into pink after the KOH solution is added. Meanwhile, the indole test also shows a negative result. This result is obtained after the *reagent* Kovac is added, which signify by the formation of a yellow ring. The existence of indole is detected by Kovac reagent and the formation of a red ring.

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CONCLUSION

This study concludes that chitinolytic bacteria isolated from blue swimmer crab's cell (*Portunus pelagicus*) genus of Pseudomonas. The highest chitinase activity is obtained from the supernatant culture obtained in the 24th hour, in which enzyme activity value is 0.149 U/mL as a good potential to degradable chitin ability.

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Chitinase activity potential and identification of chitinolytic bacteria isolated of swimmer crab's cell

Rieny Sulistijowati[1]*, Sudin[1], Rita Marsuci Harmain[1]
(Universitas Negeri Gorontalo, Faculty of Fishery and Marine Science, Departement of Fishery Product Technology, Central City of Gorontalo, 96128, Indonesia)

Abstract: This study is aimed at investigating the chitinase enzyme activity produced by chitinolytic bacteria from the skin of blue swimmer crab (*Portunus pelagicus*) and identification of the genus isolate. This study consists of two stages: firstly, the qualitative and quantitative activity of the chitinase enzyme; and secondly, biochemical identification of the bacteria. The quantitative chitinase enzyme activity is measured using the UV-Vis spectrophotometer UV-Vis at the wavelength at 660 nm. The chitinase enzyme is obtained from the isolation of chitinolytic bacteria cultured within a media to grow solid chitin, which contains colloidal chitin substrate as chitinase inductor at the Temperature of 30° C. The highest chitinolytic activity is obtained from the 24 hours supernatant culture, with a value of enzyme activity at 0.149 U/mL. Macroscopic and microscopic identification shows that the chitinolytic bacteria isolate R1, whereas the biochemical cell shows the characteristics of the genus *Pseudomonas.*

Keywords: Biodegradable, Chitinase, Spectrophotometer, *Portunus pelagicus*, *Pseudomonas*.

1 Introduction[1]

The on earth chitin is among the most abundant biomass present. Chitinase plays an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. The implementation of biotechnology toward chitin, which keeps progressing, is the utilization of enzymes from microorganisms for biodegradation. In biodegradation, an enzyme derived from microorganisms breaks large molecule or chitin polymer into utilizable products. In general, types of an enzyme that degraded the chitin are chitinase enzyme Bhattacharya, et al., [1]

Microorganisms that degraded chitin, in general, are those derived from bacteria group. Chitinase enzyme produced by chitinolytic bacteria has the potential to degrade chitin due to the existence of the chitinase enzyme, which enables the conversion of abundantly available chitin into usable products. The bacteria that produce chitinase enzyme or chitinolytic bacteria can be found within the habitat that contains a high level of chitin, such as in the cell of the blue swimmer crab. Blue swimmer crab's cell (*Portunus pelagicus*) can be obtained from the processing waste or fresh. Chitinase enzyme application can be informed of enzymatic production of chitin. The chitin can be produced enzymatically and chemically. The enzymatic method uses enzymes or bacteria for deproteinization by adding enzyme or by the involvement of chitinase to degrade chitin. Meanwhile, the chemical process is through demineralization by adding acid or strong acids, such as HCl and NaOH Younes et al., [2]

A study by Arbia et al.[3] , which isolate chitinolytic bacteria to produce several bacteria, one of them is *Pseudomonas aeruginosa* bacteria isolated from crab's cell. Purkan[4] production of chitinase enzyme from *Aspergillus niger* utilizing the blue swimmer crab's waste as inducer. Oh et al.[5] Protease produced by *Pseudomonas aeruginosa* K-187, the highest protease activity was as high as 21.2 U/ml, 10-fold that (2.2 U/ml) obtained before optimization. In common with all enzymes, external factors such as Temperature, pH and type of media are important for the activity, catalytic efficiency, stability and proper functioning of proteases Homaei et al., [6] . Chitinase activity of isolates chitinolytic bacteria can degrade different chitins.

The needs for the chitin derivatives are increasing. Thus, research on chitinase enzyme activity through the isolation of bacteria from the blue swimmer crab is needed. Observe primarily that two isolates chitinolytic bacteria from the skin of blue swimmer crab in Katialada village of Kwandang sub-district of North Gorontalo Regency, Gorontalo Province of Indonesia. Sudin et al. [7], the result obtained from the purification process following 48 hours incubation in the Temperature of 30 ^{0}C shows a clear zone that formed in the colony of the bacteria. However, specific zones are only found in white and light brown-coloured bacteria.

Further, these two isolates are macroscopically and microscopically identified. The R1 isolate shows a white-coloured colony; meanwhile, the R2 isolate shows a light brown-coloured colony. Meanwhile, from the shape/form and elevation of the colony, there are no differences between isolate R1 and R2 both colonies have a circular shape and convex elevation. The result of gram staining in these two chitinolytic bacteria isolates R1, and R2 shows the Gram-negative result. This is signified by changes of colour of these two isolates into the red after the gram staining. The study shows that CI 11 of the R1 isolate has the largest Chitinolytic Index by 1, the chitinolytic index shows the ability of the microbes to degrade chitin. The more enzyme produced, the wider the clear zone produced as more chitin is degraded.

Sudin, Rita Marsuci Harmain,
***Corresponding author: Rieny Sulistijowati,** Universitas Negeri Gorontalo, Faculty of Fishery and Marine Science, Departement of Fishery Product Technology, Indonesia, Central City of Gorontalo, Sudirman Street No. 06, Postal Code 96128. Email: rienysulistijowati@ung.ac.id.

This is due to the type of bacteria, growth pattern and enzyme activity need to be known to have a good degrading ability. Therefore, this study is aimed at tests the produced chitinase activity and identification chitinase producer bacteria of blue swimmer crab.

2 Methods and material

Data obtained from this study are quantitative and qualitative data presented in the form of tables and figures.

2.1 Station and laboratory

Isolate R1 obtain from fresh blue swimmer crab's cells are obtained from the crabs' farmer in Katialada village of Kwandang sub-district of North Gorontalo Regency, Gorontalo Province of Indonesia. The identification biochemical test of the chitinolytic bacteria are carried out at the Fish Quarantine Station Laboratory Quality Control and Fisheries Product Security Class I Gorontalo Province of Indonesia, and the chitinolytic bacteria enzyme activity test are carried out at the Pharmaceutical Laboratory of Universitas Negeri Gorontalo.

2.2 Material and methods

The tools used in this study were test tube, Ose needle, vortex, measuring cup, Beaker glass, Erlenmeyer, centrifuge, centrifuge tube, *shaker*, pH meter, micropipette, micro tip, stirrer, magnetic stabilizer, petri dish, oven, crooked spoon, bunsen lamp, incubator, laminar air, analytical scales, Uv-vis spectrophotometer, and microscope.

Materials used in this study were R1 isolated of blue swimmer crab's cell, chitin, chitin colloidal (chitin, condensed HCl, NaOH, aquadest), chitin agar (chitin colloidal, KH_2PO_4, $MgSO_4.7H_2O$, yeast extract, agar, aquadest), Luria broth (yeast extract, tripton, NaCl, aquadest), nutrient agar, aluminum foil, alcohol, crystal violet, iodine solution, glucose, sucrose, lactose, maltose, mannitol, triple sugar iron agar, motility indole ornitin, oxidative/fermentative, methyl red-Voges Proskauer, methyl-red, and sterile liquid paraffin.

2.3 Quantitative activity of chitinase enzyme test

One dose of inoculum is added into 100 mL of production medium (similar composition to a solid medium, but without agar) then incubated in the Temperature of 30 ^{0}C and centrifuged in the speed of 170 rpm. Every three hours, 2 mL of cell culture is sampled for 33 hours. Then centrifuged in the Temperature of 4 ºC using 10.000 rpm speed for 10 minutes, the formed supernatant is the raw extract of chitinase enzyme. The absorbance is then measured using Spectrophotometer U.V./Vis in λ 660 nm Purkan et al, [8], the sample is carried out twice repetitions.

2.4 Biochemical test

The biochemical test is carried out to identify and classify bacteria into their group of taxonomy. The principle of this biochemical test is if the bacteria are cultured in several media, the bacteria will show macroscopic differences in their growth Cappuccino and Sherman[9], Adyta et al., [10] were; Carbohydrate fermentation test to find out the bacteria's ability in fermenting carbohydrate by preparing the carbohydrate broth which consists of glucose, sucrose, maltose, and mannitol; MR-VP (methyl red-Voges Proskauer) test to inoculate bacteria into a medium, which incubated in the Temperature of 30 ºC for 24 hours by adding methyl red reagent and KOH, to observe the bacteria ability in producing the mix acid and acetylenes; Citrate test by inoculating bacteria into a medium and incubated in the Temperature of 30 ºC for 24 hours by adding BTB (bromothymol blue) reagent, then observe the ability of the bacteria to use citrate as the only source of carbon; H_2S (Hydrogen Sulfide) test to inoculate the bacteria into SIM (sulfide indole motility) which incubated for 24-48 hours in the Temperature of 30 ºC, then observe the ability of the bacteria to produce H_2S which signify by the existence of black sediment; O/F (oxidation/fermentation) test by inoculating the bacteria into the O/F medium, which incubated for 24 hours in the Temperature of 30 ºC, then observe the ability of the bacteria to use carbohydrate through fermentation or oxidation; TSIA (triple sugar iron agar) test by inoculating bacteria into TSIA media, which incubated for 24-48 hours in the Temperature of 30ºC, then observe the ability of the bacteria in fermenting glucose, lactose, and sucrose; Indole test by inoculating bacteria into indole media, which incubated for 24 hours in the Temperature of 30 ºC, then observe the ability of bacteria in degrading the tryptophan amino acid in the medium.

3 Results and discussion

3.1 Quantitative activity of the chitinolytic bacteria enzyme

Data and standard deviation presented in Table 1.

Replication	Incubation (hours)											
	0	3	6	9	12	15	18	21	24	27	30	33
1	0	0.04	0.05	0.04	0.13	0.15	0.06	0.08	0.14	0.115	0.1	0.09
2	0	0.025	0.03	0.05	0.14	0.17	0.08	0.1	0.16	0.135	0.12	0.07
Mean	0	0.0325	0.04	0.045	0.135	0.16	0.07	0.09	0.15	0.125	0.11	0.08
Standard deviation	0	0.01061	0.01414	0.00707	0.0070711	0.01414	0.01414	0.01414	0.01414	0.01414	0.01414	0.01414

The activity enzyme of chitinolytic bacteria is presented in Figure 1.

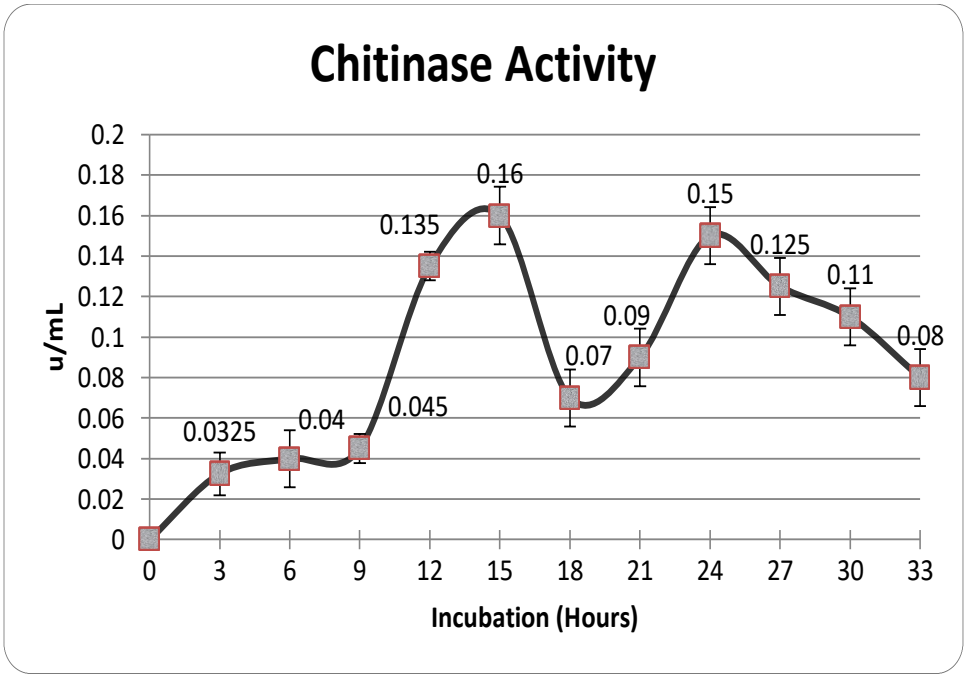

Figure 1 Chitinase Enzyme Activity Curve

The activity of the chitinolytic bacteria enzyme is essential to be known to find out the ability of the bacteria to produce the enzyme in 33 hours with an interval of 3 hours. There are several increasing and decreasing stages in chitinase activity. The first inclination happens during the incubation time of 0 hours to 12 hours. The second increase of the enzyme activity shows that the substrate is starting to be hydrolyzed to produce the chitinase enzyme. Hence, bacteria can digest nutrition. Patil et al, [11] wrote that bacteria produce extracellular chitinase to take on nutrition. Following this inclination, there is the first declining phase on the 15th to the 18th hour of incubation. The decrease of this enzyme activity is due to other compounds (aside from N-Acetyl glucosamine) that triggers the decrease of enzyme production.

This phenomenon is due to the existence of other chitin-degrading enzymes produced by the bacteria. Fukamizo[12] argues that colloidal chitin also can be hydrolyzed by deacetylating chitin produced by chitosan and chitosanase which produce chitobiose. Following this declining phase, the chitinase activity climbs up in the incubation time of a 21st hour to its highest chitinase activity that can be obtained from the supernatant culture in the incubation of time of a 24th hour, which stated by the value of enzyme activity by 0.149 U/mL. One unit of chitinase enzyme activity is defined as several enzymes needed to release one mmol NAG/minute. This result is different from the result of the enzymes activity test carried out by Purkan et al[8] who found that that the highest enzyme activity is in the 18th hour of incubation time, which started with the value of enzyme activity by 0.3850 U/mL. Also, Orinda et al, [13] argue that the ability of the bacteria to produce chitinase highly varied. Factors such as different types of bacteria, the growth rate of each isolate in the medium, or laboratory treatment during the experiment can be factors that influence variation in the produced enzyme activity.

The rebound of enzyme activity shows that there is more of the substrate being hydrolyzed. The chitinase enzyme activity is steadily increasing until it reaches optimum incubation time. Following the reach of this optimum incubation time, the enzyme activity decreases due to the accumulation of hydrolyzed products, which can further inhibit the enzyme activity. This is characterized by the decrease of enzyme activity on the incubation time of hour 27 to hour 33. Fukamizo [12] argues that this decrease of chitinase enzyme activity after the optimum incubation time is due to the changes in the state of the enzyme ion and the state of substrate ion which caused denaturation of enzyme which followed by the loose of enzyme catalytic activity. Besides, there are also change in the tertiary structure of the enzyme due to denaturation, which made the hydrophobic amino acid group within the enzyme to come into contact with water, thus, the solubility of the enzyme weakens. The decrease of chitinase solubility causes a gradual decrease in enzyme activity.

Chitinolytic bacteria isolates show unstable chitinase activity (fluctuate). Orinda et al., [13] suggest that this may be due to the isolate that produces the chitinase at the beginning of its growth. In line with the utilization of nutrition for growth, it is also suspected that chitinase is also used by bacteria as a source of protein, thus its chitinase activity decreases.

The decrease of enzyme activity can also be caused by factors such as Temperature, pH, substrate and biomass during treatment in the laboratory. The Temperature has two main influences on the reaction and the denaturation. The influence of reaction toward the enzyme is that the increase of Temperature will accelerate the reaction process, while the decrease in the Temperature will cause the reaction to slow down. When the Temperature reaches a certain limit, it will cause denaturation. Besides, when the pH of the environment is too acid or base, enzyme denaturation can also happen. Reaction speed catalyzed by the enzyme is highly influenced by substrate concentration. In the low level of substrate concentration, reaction speed by catalyzed by the enzyme can also be very low. In reverse, reaction speed will increase along with the increase of substrate concentrate up to certain points that is the maximum reaction speed limit. When this saturated point of the enzyme has been reached, it will not function properly. Lastly, the number of bacteria inoculum (biomass) inserted into the media also strongly influence the enzyme activity.

3.2 Biochemical test

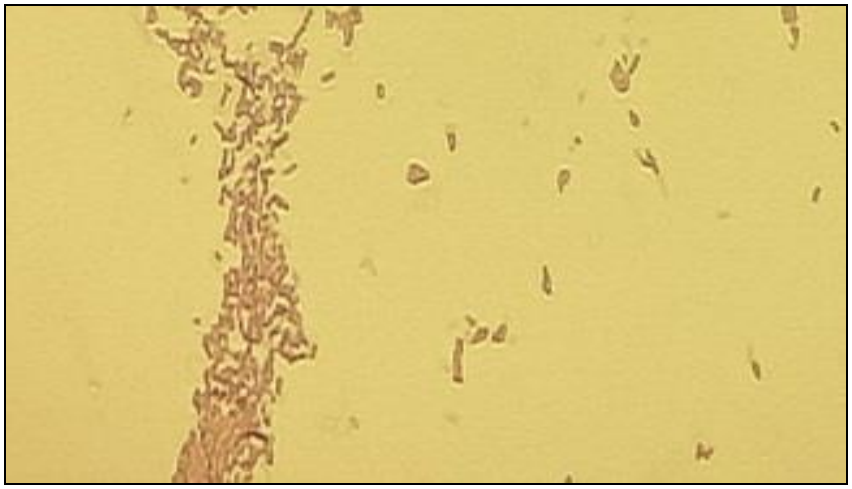

Figure 2 Isolate R1 (10x100 magnification)

The biochemical test toward the characteristics of chitinolytic bacteria is carried out by fermenting bacteria in the various sources of nutrition. The biochemical test result of chitinolytic bacteria is presented in Table 1.

Fermentation test in several types of carbohydrate (glucose, maltose, sucrose, mannitol, lactose) shows that all fermentation reaction is negative. This is characterized by the unchanging red colour of the carbohydrate media. Aditi et al., [10] argue that when the colour of the medium in the carbohydrate test turns into yellow, it means that the colony forms acid from that carbohydrate.

A citrate test is carried out to find out the ability of the chitinolytic bacteria isolates to utilize citrate as the only source of carbon and energy. When a microorganism can use citrate, there will be an increase of pH and change in the colour of the media into blue colour. In this study, the citrate test reveals that the chitinolytic bacteria cannot utilize citrate as the only source of carbon. This is shown by the unchanging green colour of the media, which means that the test result is negative. Hemraj et al., [14] argue that positive test results in the citrate test are shown when the colour changes from green to blue.

Table 2 Biochemical characteristics of chitinolytic bacteria isolate R1

No	Test	Results
1	Glucose fermentation	Negative
2	Sucrose fermentation	Negative
3	Lactose fermentation	Negative
4	Maltose fermentation	Negative
5	Mannitol fermentation	Negative
6	Citrate Use	Negative
7	Sulfide Indole Motility	Negative
8	Triple Sugar Iron Agar	Alkaline/Alkaline
9	Methyl Red Reaction	Negative
10	Voges Proskauer Reaction	Negative
11	Indole production	Negative
12	Oxidase/Fermentative activity	Negative

Further, the H_2S test result in SIM is negative. This negative result is reached when microorganism has no ability to hydrolyzed heavy metal within the media. H_2S is produced by several types of microorganisms, which can break or degrade amino acid within the sulfur (S). The existence of H_2S can be observed by adding several crystals of heavy metals into the media.

The reaction observable in the TSIA test shows a red colour, which means that there is no change of colour in both vertical and slight agar. This indicates that the bacteria are unable to ferment sugar. Amano et al., [15] opine that in the vertical agar if the bacteria can ferment glucose, the colour of the media will change from red to yellow. Whereas in slight agar, if the bacteria can ferment lactose and sucrose, the colour of the media will change into yellow, meanwhile, when there is no fermentation process of lactose and sucrose, the colour will not change.

Methyl red test also reveals a negative result. This is shown by the unchanging color of the media which does not change into yellow even after the addition of methyl red reagent. Hemraj et al., [14] wrote that the red colour signifies the positive test result, and if the colour of the broth is yellow, then the result of the test is negative. Similarly, the Voges Proskauer test also shows a negative result. This is evident after the addition of the KOH solution; the colour does not change. The V.P. test will be stated as positive when there is a form of acid,

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To: Dr Lis Melissa Yapanto <lizrossler@ung.ac.id>
Cc: Rieny Sulistijowati <rienysulistijowati@ung.ac.id>

Dr Rieny Sulistijowati:

We have reached a decision regarding your submission to International Journal of Agricultural and Biological Engineering, "Chitinase activity of chitinolytic bacteria Pseudomonas sp. from blue swimmer crab's cell (Portunus pelagicus)".

Our decision is to: Major Revision.

If you wish to review and resubmit the manuscript for technical review, please do so within 20 days.

Peng Yankun
China Agricultural University
Phone 010-62737703
Fax 010-62737703
ypeng@cau.edu.cn

Ph.D., Prof., Engineering College, China Agricultural University, Beijing.
Email: ypeng@cau.edu.cn

Reviewer 1:

Review Summary

Rate the overall quality of this paper on the following scale (Select):
2 = Fair

What is your recommendation for this paper? Please select one::
Major revision requiring re-review

Technical Quality: In this section, please evaluate the technical merit of the paper, aside from presentation issues.

Would at least 10% of the agricultural and biological engineering community be interested in this paper?:
A = Yes, it would be

Would this paper stimulate further work?:
A = Yes, it would be

Does this paper present original and creative ideas?:
A = Yes, it does

Are the results of this paper important?:
A = Yes, they are important

Is this paper technically sound? (If not, please provide details in the comments section):
A = Yes, it is

If the paper presents practical material, is it sufficiently well explained that a competent graduate student equipped with the references could write an implementation (repeatability)?:
B = No, it is not

Presentation Quality: In this section, please evaluate the presentation of the paper, aside from technical issues.

Is the title appropriate?:
A = Yes, it is

Is the abstract acceptable?:
A = Yes, it is

Is the bibliography complete?:
A = Yes, it is

Is the English acceptable?:
B = No, it is not

Is the number of figures appropriate? If not, would you recommend more or less figures?:
B = No, it is not

Is the organization of the paper acceptable? If not, recommend specific changes.:
B = No, it is not

Aside from technical content, rate the quality of the presentation::
1 = Poor

Comments

This section is the most valuable part of the review for the author(s), who are extremely interested in how you formed your opinion of this paper. Please provide specific comments that will help the author(s) understand your review, and possibly prepare a revision. Use all the space you need (The input box can be expanded by dragging the lower corner).

General Comments::

1. Overall detailed manuscript discussing the procedure and results of investigating the chitinase enzyme activity produced by chitinolytic bacteria from the skin of the blue swimmer's crab.

Specific comments::

1. In the introduction, it needs to be mentioned why this study is different and novel from similar studies that have been cited.

2. Under the Tools and Ingredients section, please summarize them in a table. Currently, listing all tools are ingredients makes the manuscript pretty verbose.

3. The overall English quality of the manuscript is not upto the mark, and major revision is required.

4. The figures are of poor quality.

5. Under 'Quantitative Activity of Chininase Enzyme Test', a wide variety of biochemical tests are given. Not sure if all of them are relevant to this manuscript.

6. The UV spectrometer data is not shown.

Reviewer 2:

Review Summary

Rate the overall quality of this paper on the following scale (Select):

2 = Fair

What is your recommendation for this paper? Please select one::

Major revision requiring re-review

Technical Quality: In this section, please evaluate the technical merit of the paper, aside from presentation issues.

Would at least 10% of the agricultural and biological engineering community be interested in this paper?:

A = Yes, it would be

Would this paper stimulate further work?:

B = No, it would not

Does this paper present original and creative ideas?:

B = No, it does not

Are the results of this paper important?:

A = Yes, they are important

Is this paper technically sound? (If not, please provide details in the comments section):

A = Yes, it is

If the paper presents practical material, is it sufficiently well explained that a competent graduate student equipped with the references could write an implementation (repeatability)?:

A = Yes, it is

Presentation Quality: In this section, please evaluate the presentation of the paper, aside from technical issues.

Is the title appropriate?:

A = Yes, it is

Is the abstract acceptable?:

B = No, it is not

Is the bibliography complete?:

B = No, it is not

Is the English acceptable?:

B = No, it is not

Is the number of figures appropriate? If not, would you recommend more or less figures?:

A = Yes, it is

Is the organization of the paper acceptable? If not, recommend specific changes.:

B = No, it is not

Aside from technical content, rate the quality of the presentation::

1 = Poor

Comments

This section is the most valuable part of the review for the author(s), who are extremely interested in how you formed your opinion of this paper. Please provide specific comments that will help the author(s) understand your review, and possibly prepare a revision. Use all the space you need (The input box can be expanded by dragging the lower corner).

General Comments::

This manuscript investigated the chitinase enzyme activity produced by chitinolytic bacteria from the skin of blue swimmer crab (Portunus pelagicus) and identified the genus isolate. The experimental part is complete while the technologies and methods involved are not new now. The methodologies and results are clearly described in the manuscript, however, the novelty lacks. In addition, the language needs improvement throughout the manuscript.

Specific comments::

Abstract: “in the wavelength of 660 nm” needs to be changed to “at the wavelength of 660 nm”, and also other places similar in the MS. “Chitinase enzyme is obtained from the isolation of chitinolytic bacteria cultured within a media to grow solid chitin, which contains colloidal chitin substrate as chitinase inductor in the temperature of 30 °C” needs to be rephrased. “in the temperature of ” needs to be changed to “at the temperature of ”, and also other places similar in the MS.

Introduction: The authors did not list the reported studies that are relevant to this study. Based on these studies, the context why this study needs to be conducted should be given clearly in this part.

RESEARCH METHOD: This subtitle needs to be changed to “Materials and Methods”.

Research site: It is not necessary to present. However, if the authors would like to present, the city and country information needs to be added for the station and lab.

Preparation of Blue Swimmer Crab’s Cell Sample (P.pelagicus): “Fresh blue swimmer crab’s cells are obtained from” needs to be changed to “…were obtained from”.
“This sample stored into a cool box with ice cubes layered with sealed and sterilized plastics. This is in order for the sample to not have direct contact with the ice blocks.”- Grammar mistakes, please correct.

Preparation of Colloidal Chitin: “8.000 rpm for 20 minutes” corrected to “8,000 rpm for 20 minutes”, and also other places similar in the MS.

III. FINDINGS AND DISCUSSION: A general way for this subtitle is “Results and Discussion”

Figure 4: How many repetitions were conducted for each time point? The standard deviation data needs to be added to this figure.

Table 1: “0,4” needs to be changed to “0.4”. Similar changes are needed all through the table.
Please use the same precision level for the data presented in this table.

Conclusion: The potential impact of the obtained results needs to be discussed here. Also, future perspective regarding lacks.

Prof. Wang Yingkuan, PhD

Editor-in-chief, IJABE (SCI, IF1.349)
Call: 86-10-59197090, 59197088
Add: No.41, Maizidian Street, Chaoyang District, Beijing 100125, China
Email: ijabe@sina.com, ijabe@outlook.com https://www.ijabe.org

BalasTeruskan

Chitinase activity potential and identification of chitinolytic bacteria isolated of swimmer crab's cell

Rieny Sulistijowati*, Sudin, Rita Marsuci Harmain
(Universitas Negeri Gorontalo, Faculty of Fishery and Marine Science, Departement of Fishery Product Technology, Central City of Gorontalo, 96128, Indonesia)

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Yes…Full name only S

Abstract: This study is aimed at investigating the chitinase enzyme activity produced by chitinolytic bacteria from the skin of blue swimmer crab (*Portunus pelagicus*) and identification of the genus isolate. This study consists of two stages: firstly, the qualitative and quantitative activity of the chitinase enzyme; and secondly, biochemical identification of the bacteria. The quantitative chitinase enzyme activity is measured using the UV-Vis spectrophotometer UV-Vis at the wavelength at 660 nm. The chitinase enzyme is obtained from the isolation of chitinolytic bacteria cultured within a media to grow solid chitin, which contains colloidal chitin substrate as chitinase inductor at the temperature of 30℃. The highest chitinolytic activity is obtained from the 24 h supernatant culture, with a value of enzyme activity at 0.149 U/mL. Macroscopic and microscopic identification showed that the chitinolytic bacteria isolate R1, whereas the biochemical cell shows the characteristics of the genus *Pseudomonas*.

Keywords: biodegradable, chitinase, spectrophotometer, *Portunus pelagicus*, *Pseudomonas*.

1 Introduction[1]

The on earth chitin is among the most abundant biomass present. Chitinase plays an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. The implementation of biotechnology toward chitin, which keeps progressing, is the utilization of enzymes from microorganisms for biodegradation. In biodegradation, an enzyme derived from microorganisms breaks large molecule or chitin polymer into utilizable products. In general, types of an enzyme that degraded the chitin are chitinase enzyme[1].

Microorganisms that degraded chitin, in general, are those derived from bacteria group. Chitinase enzyme produced by chitinolytic bacteria has the potential to degrade chitin due to the existence of the chitinase enzyme, which enables the conversion of abundantly available chitin into usable products. The bacteria that produce chitinase enzyme or chitinolytic bacteria can be found within the habitat that contains a high level of chitin, such as in the cell of the blue swimmer crab. Blue swimmer crab's cell (*Portunus pelagicus*) can be obtained from the processing waste or fresh. Chitinase enzyme application can be informed of enzymatic production of chitin. The chitin can be produced enzymatically and chemically. The enzymatic method uses enzymes or bacteria for deproteinization by adding enzyme or by the involvement of chitinase to degrade chitin. Meanwhile, the chemical process is through demineralization by adding acid or alkali, such as HCl and NaOH[2].

A study by[3], which isolate chitinolytic bacteria to produce several bacteria, one of them is *Pseudomonas aeruginosa* bacteria isolated from crab's cell. The production of chitinase enzyme from *Aspergillus niger* utilizing the blue swimmer crab's waste as inducer[4]. Protease produced by *Pseudomonas aeruginosa* K-187, the highest protease activity was as high as 21.2 U/mL, 10-fold that (2.2 U/mL) obtained before optimization[5]. In common with all enzymes, external factors such as temperature, pH and type of media are important for the activity, catalytic efficiency, stability and proper functioning of proteases[6]. Chitinase activity of isolates chitinolytic bacteria can degrade different chitins.

The needs for the chitin derivatives are increasing. Thus, researches on chitinase enzyme activity through the isolation of bacteria from the blue swimmer crab is needed. Observe primarily that two isolates chitinolytic bacteria from the skin of blue swimmer crab in Katialada village of Kwandang sub-district of North Gorontalo Regency, Gorontalo Province of Indonesia. The results obtained from the purification process following 48 h incubation in the temperature of 30℃ shows a clear zone that formed in the colony of the bacteria[7]. However, specific zones are only found in white and light brown-coloured bacteria.

Furthermore, these two isolates are macroscopically and microscopically identified. The R1 isolate shows a white-coloured colony; meanwhile, the R2 isolate shows a light brown-coloured colony. Meanwhile, from the shape/form and elevation of the colony, there are no differences between isolates R1 and R2 both colonies have a circular shape and convex elevation. The result of gram staining in these two chitinolytic bacteria isolates R1, and R2 shows the gram-negative result. This is signified by changes of colour of these two isolates into the red after the gram staining. The study shows that CI 11 of the R1 isolate has the largest Chitinolytic Index by 1, the chitinolytic index shows the ability of the microbes to degrade chitin. The more enzyme produced, the wider the clear zone produced as more chitin is degraded.

This is due to the type of bacteria, growth pattern and enzyme activity need to be known to have a good degrading ability. Therefore, this study is aimed at tests the produced chitinase activity and identification chitinase producer bacteria of blue swimmer crab.

Biographies: Sudin, Junior Scientist, research interest: marine biotechnology, Email: sudynsultra@gmail.com; **Rita Marsuci Harmain**, research assistant, research interest: marine microbiology, Email: rmarsuci@yahoo.com.
*Corresponding author: **Rieny Sulistijowati**, Associate Professor, research interest: biotechnology process. Universitas Negeri Gorontalo, Faculty of Fishery and Marine Science, Departement of Fishery Product Technology, Indonesia, Central City of Gorontalo, Sudirman Street No. 06, Postal Code 96128. Tel: +62 (0435) 821125 Email: rienysulistijowati@ung.ac.id.

2 Methods and material

2.1 Station and laboratory

Isolate R1 obtain from fresh blue swimmer crab's cells are obtained from the crabs' farmer in Katialada village of Kwandang sub-district of North Gorontalo Regency, Gorontalo Province of Indonesia. The identification biochemical test of the chitinolytic bacteria are carried out at the Fish Quarantine Station Laboratory Quality Control and Fisheries Product Security Class I Gorontalo Province of Indonesia, and the chitinolytic bacteria enzyme activity test are carried out at the Pharmaceutical Laboratory of Universitas Negeri Gorontalo.

2.2 Materials and methods

The tools used in this study were test tube, ose needle, vortex, measuring cup, beaker glass, erlenmeyer, centrifuge, centrifuge tube, shaker, pH meter, micropipette, micro tip, stirrer, magnetic stabilizer, petri dish, oven, crooked spoon, bunsen lamp, incubator, laminar air, analytical scales, Uv-vis spectrophotometer, and microscope.

Materials used in this study were R1 isolated of blue swimmer crab's cell, chitin, chitin colloidal (chitin, condensed HCl, NaOH, aquadest), chitin agar (chitin colloidal, KH_2PO_4, $MgSO_4.7H_2O$, yeast extract, agar, aquadest), Luria broth (yeast extract, tripton, NaCl, aquadest), nutrient agar, aluminum foil, alcohol, crystal violet, iodine solution, glucose, sucrose, lactose, maltose, mannitol, triple sugar iron agar, motility indole ornitin, oxidative/fermentative, methyl red-voges proskauer, methyl-red, and sterile liquid paraffin.

2.3 Quantitative activity of chitinase enzyme test

One dose of inoculum is added into 100 mL of production medium (similar composition to a solid medium, but without agar) then incubated in the temperature of 30°C and centrifuged in the speed of 170 r/min. Every three hours, 2 mL of cell culture is sampled for 33 h. Then centrifuged in the temperature of 4°C using 10.000 r/min speed for 10 min, the formed supernatant is the raw extract of chitinase enzyme. The absorbance is then measured using spectrophotometer UV/Vis in wavelength of 660 nm[8], the sample is carried out twice repetitions.

2.4 Biochemical test

The biochemical test is carried out to identify and classify bacteria into their group of taxonomy. The principle of this biochemical test is if the bacteria are cultured in several media, the bacteria show macroscopic differences in their growth[9,10]. Carbohydrate fermentation test to find out the bacteria's ability in fermenting carbohydrate by preparing the carbohydrate broth which consists of glucose, sucrose, maltose, and mannitol; MR-VP (methyl red-Voges Proskauer) test to inoculate bacteria into a medium, which incubated in the temperature of 30°C for 24 h by adding methyl red reagent and KOH, to observe the bacteria ability in producing the mix acid and acetylenes; Citrate test by inoculating bacteria into a medium and incubated in the temperature of 30°C for 24 h by adding bromothymol blue (BTB) reagent, then observe the ability of the bacteria to use citrate as the only source of carbon; H_2S test to inoculate the bacteria into sulfide indole motility (SIM) which incubated for 24-48 h in the temperature of 30°C, then observe the ability of the bacteria to produce H_2S which signify by the existence of black sediment; oxidation/fermentation (O/F) test by inoculating the bacteria into the O/F medium, which incubated for 24 h in the temperature of 30°C, then observe the ability of the bacteria to use carbohydrate through fermentation or oxidation; TSIA (triple sugar iron agar) test by inoculating bacteria into TSIA media, which incubated for 24-48 h in the temperature of 30°C, then observe the ability of the bacteria in fermenting glucose, lactose, and sucrose; Indole test by inoculating bacteria into indole media, which incubated for 24 h in the temperature of 30°C, then observe the ability of bacteria in degrading the tryptophan amino acid in the medium.

3 Results and discussion

3.1 Quantitative activity of the chitinolytic bacteria enzyme

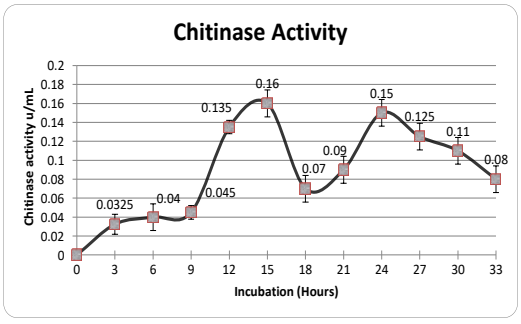

Figure 1 Chitinase activity curve from *Pseudomonas*

The activity of the chitinolytic bacteria enzyme is essential to be known to find out the ability of the bacteria to produce the enzyme in 33 h with an interval of 3 h. There are several increasing and decreasing stages in chitinase activity. The first inclination happens during the incubation time of 0 to 12 h. The second increase of the enzyme activity shows that the substrate is starting to be hydrolyzed to produce the chitinase enzyme. Hence, bacteria could digest nutrition. Patil et al[11] found that bacteria produce extracellular chitinase to take on nutrition.

Following this inclination, there is the first declining phase on the 15th to the 18th hour of incubation. The decrease of this enzyme activity is due to other compounds (aside from N-Acetyl glucosamine) that triggers the decrease of enzyme production.

This phenomenon is due to the existence of other chitin-degrading enzymes produced by the bacteria. Colloidal chitin also can be hydrolyzed by deacetylating chitin produced by chitosan and chitosanase which produce chitobiose[12]. Following this declining phase, the chitinase activity climbs up in the incubation time of the 21st hour to its highest chitinase activity that can be obtained from the supernatant culture in the incubation of time of the 24th hour, which stated by the value of enzyme activity by 0.149 U/mL. One unit of chitinase enzyme activity is defined as several enzymes needed to release 1 mmol NAG/min. This result is different from the result of the enzymes activity test carried out by Purkan et al[8] who found that the highest enzyme activity is in the 18th hour of incubation time, which started with the value of enzyme activity by 0.3850 U/mL. Futhermore, the ability of the bacteria to produce chitinase highly varied. Factors such as different types of bacteria, the growth rate of each isolate in the medium, temperature, pH or laboratory treatment during the experiment can be factors that influence variation in the produced enzyme activity[13,14]. Chitinase activity was 0.213 and 0.219 U/ml respectively of PBK 2 and SA 1.2 isolates from shrimp waste. Based on 16S rDNA sequences, isolate of PBK 2 was identified as *Acinetobacter johnsonii* 3-1, whereas SA 1.2 was identified as *Bacillus amyloliquefaciens* GR53 with 99.78% similarity[15].

The rebound of enzyme activity shows that there is more of the substrate being hydrolyzed. The chitinase enzyme activity is steadily increasing until it reaches optimum incubation time. Following the reach of this optimum incubation time, the enzyme activity decreases due to the accumulation of hydrolyzed products, which can further inhibit the enzyme activity. This is characterized by the decrease of enzyme activity on the incubation time of hour 27 to hour 33. The decrease of chitinase enzyme activity after the optimum incubation time is due to the changes in the state of the enzyme ion and the state of substrate ion which caused denaturation of enzyme which followed by the loose of enzyme catalytic activity[12]. Besides, there are also change in the tertiary structure of the enzyme due to denaturation, which made the hydrophobic amino acid group within the enzyme to come into contact with water, thus, the solubility of the enzyme weakens. The decrease of chitinase solubility causes a gradual decrease in enzyme activity.

Chitinolytic bacteria isolates showed unstable chitinase activity (fluctuate). This may be due to the isolate that produces the chitinase at the beginning of its growth[13]. In line with the utilization of nutrition for growth, it is also suspected that chitinase is also used by bacteria as a source of protein, thus its chitinase activity decreases.

The decrease of enzyme activity can also be caused by factors such as temperature, pH, substrate and biomass during treatment in the laboratory. The temperature has two main influences on the reaction and the denaturation. The influence of reaction toward the enzyme is that the increase of temperature will accelerate the reaction process, while the decrease in the temperature will cause the reaction to slow down. When the temperature reaches a certain limit, it will cause denaturation. Besides, when the pH of the environment is too acid or base, enzyme denaturation can also happen. Reaction speed catalyzed by the enzyme is highly influenced by substrate concentration. In the low level of substrate concentration, reaction speed by catalyzed by the enzyme can also be very low. In reverse, reaction speed will increase along with the increase of substrate concentrate up to certain points that is the maximum reaction speed limit. When this saturated point of the enzyme has been reached, it will not function properly. Lastly, the number of bacteria inoculum (biomass) inserted into the media also strongly influence the enzyme activity.

3.2 Biochemical test

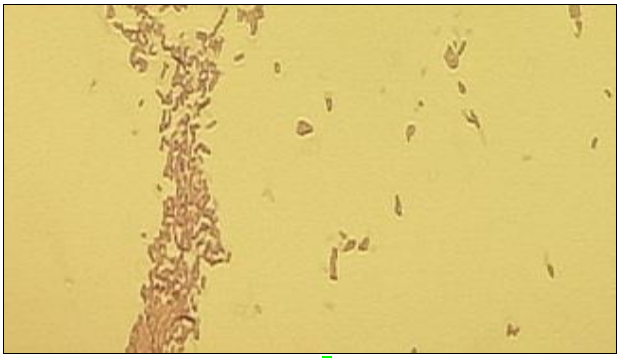

Figure 2 Isolate R1 (10×100 magnification)

The biochemical test toward the characteristics of chitinolytic bacteria is carried out by fermenting bacteria in the various sources of nutrition. The biochemical test result of chitinolytic bacteria is presented in **Table 1**.

Fermentation test in several types of carbohydrate (glucose, maltose, sucrose, mannitol, lactose) shows that all fermentation reaction is negative. This is characterized by the unchanging red colour of the carbohydrate media. When the colour of the medium in the carbohydrate test turns into yellow, it means that the colony forms acid from that carbohydrate[10].

A citrate test is carried out to find out the ability of the chitinolytic bacteria isolates to utilize citrate as the only source of carbon and energy. When a microorganism can use citrate, there will be an increase of pH and change in the colour of the media into blue colour. In this study, the citrate test reveals that the chitinolytic bacteria cannot utilize citrate as the only source of carbon. This is shown by the unchanging

green colour of the media, which means that the test result is negative. Positive test results in the citrate test are shown when the colour changes from green to blue[16].

Table 1 Biochemical characteristics of chitinolytic bacteria isolate R1

No	Test	Results
1	Glucose fermentation	Negative
2	Sucrose fermentation	Negative
3	Lactose fermentation	Negative
4	Maltose fermentation	Negative
5	Mannitol fermentation	Negative
6	Citrate use	Negative
7	Sulfide indole motility	Negative
8	Triple sugar iron agar	Alkaline/Alkaline
9	Methyl red reaction	Negative
10	Voges Proskauer reaction	Negative
11	Indole production	Negative
12	Oxidase/Fermentative action	Negative

Further, the H_2S test result in SIM is negative. This negative result is reached when microorganism has no ability to hydrolyzed heavy metal within the media. H_2S is produced by several types of microorganisms, which can break or degrade amino acid within the sulfur (S). The existence of H_2S can be observed by adding several crystals of heavy metals into the media.

The reaction observable in the TSIA test shows a red colour, which means that there is no change of colour in both vertical and slight agar. This indicates that the bacteria are unable to ferment sugar. In the vertical agar if the bacteria can ferment glucose, the colour of the media will change from red to yellow[17]. Whereas in slight agar, if the bacteria can ferment lactose and sucrose, the colour of the media will change into yellow, meanwhile, when there is no fermentation process of lactose and sucrose, the colour will not change.

Methyl red test also reveals a negative result. This is shown by the unchanging color of the media which does not change into yellow even after the addition of methyl red reagent. The red colour signifies the positive test result, and if the colour of the broth is yellow, then the result of the test is negative[16]. Similarly, the Voges Proskauer test also shows a negative result. This is evident after the addition of the KOH solution; the colour does not change. The V.P. test will be stated as positive when there is a form of acid, which signifies by the changes of medium colour into pink after the KOH solution is added. Meanwhile, the indole test also shows a negative result. This result is obtained after the reagent Kovac is added, which signify by the formation of a yellow ring. The existence of indole is detected by Kovac reagent and the formation of a red ring.

The objective of the oxidizing fermentative test is to find out the oxidation and fermentation characteristics of bacteria toward glucose. Based on the result of the study on the O/F test, it does not show either oxidation or fermentation. This is evident when the media, either without liquid paraffin or without paraffin at all, do not change colour. Bacteria are said to be fermentative when both inoculated media change colour into yellow. Bacteria are oxidative when tube sealed with no paraffin changes colour into yellow and the tube sealed with paraffin does not change colour[18].

The morphology test result (macroscopic and microscopic tests) of chitinolytic bacteria have bar cell and gram-negative[9]. The biochemical test of the chitinolytic bacteria consists of carbohydrate, citrate, sulfide indole motility, triple sugar iron agar, MR-VP, indole and O/F test should obtain negative results for as indicators of a genus of Pseudomonas. Several studies to determine chitinolytic bacteria from the genus of Pseudomonas[4] that utilized blue swimmer crab's waste as an inducer to the production of chitinase enzyme from *Aspergillus niger*. A study by Arbia et al[3], which isolate chitinolytic bacteria to produce several bacteria, one of them is *Pseudomonas aeruginosa* bacteria isolated from crab's cell. Genus *Pseudomonas* generally has bar cell shape and gram-negative. A study by[19] which isolate *Pseudomonas aeruginosa* K-187 known produced two bifungtional chitinase and lysosyme. Thompson et al[20] found that *Pseudomonas aeruginosa* strain 385 produced chitinase antigen.

4 Conclusions

This study concludes that chitinolytic bacteria isolated from blue swimmer crab's cell (*Portunus pelagicus*) genus of Pseudomonas. The highest chitinase activity is obtained from the supernatant culture obtained in the 24th hour, in which enzyme activity value is 0.149 U/mL as a good potential to degradable chitin ability.

Acknowledgements

We would like to thank Fish Quarantine Station Laboratory Quality Control and Fisheries Product Security Class I Gorontalo Province of Indonesia, and Pharmaceutical Laboratory of Universitas Negeri Gorontalo support facility for this research.

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Answer: Indonesia paper

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Answer: Indonesia paper

Chitinase activity potential and identification of chitinolytic bacteria isolated of swimmer crab's cell

Rieny Sulistijowati*, Sudin, Rita Marsuci Harmain

(*Universitas Negeri Gorontalo, Faculty of Fishery and Marine Science, Department of Fishery Product Technology, Central City of Gorontalo, 96128, Indonesia*)

Abstract: This study aimed at investigating the chitinase enzyme activity produced by chitinolytic bacteria from the skin of blue swimmer crab (*Portunus pelagicus*) and identification of the genus isolate. This study consists of two stages: firstly, the qualitative and quantitative activity of the chitinase enzyme; and secondly, biochemical identification of the bacteria. The quantitative chitinase enzyme activity is measured using the UV-Vis spectrophotometer UV-Vis at the wavelength at 660 nm. The chitinase enzyme is obtained from the isolation of chitinolytic bacteria cultured within a media to grow solid chitin, which contains colloidal chitin substrate as chitinase inductor at the temperature of 30°C. The highest chitinolytic activity is obtained from the 24 h supernatant culture, with a value of enzyme activity at 0.149 U/mL. Macroscopic and microscopic identification showed that the chitinolytic bacteria isolate R1, whereas the biochemical cell shows the characteristics of the genus *Pseudomonas*.

Keywords: biodegradable, chitinase, spectrophotometer, *Portunus pelagicus*, *Pseudomonas*

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1 Introduction

The earth chitin is among the most abundant biomass present. Chitinase plays an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource. The implementation of biotechnology toward chitin, which keeps progressing, is the utilization of enzymes from microorganisms for biodegradation. In biodegradation, an enzyme derived from microorganisms breaks large molecule or chitin polymer into utilizable products. In general, types of an enzyme that degraded the chitin are chitinase enzyme [1].

Microorganisms that degraded chitin, in general, are those derived from bacteria group. Chitinase enzyme produced by chitinolytic bacteria has the potential to degrade chitin due to the existence of the chitinase enzyme, which enables the conversion of abundantly available chitin into usable products. The bacteria that produce chitinase enzyme or chitinolytic bacteria can be found within the habitat that contains a high level of chitin, such as in the cell of the blue swimmer crab. Blue swimmer crab's cell (*Portunus pelagicus*) can be obtained from the processing waste or fresh. Chitinase enzyme application can be informed of enzymatic production of chitin. The chitin can be produced enzymatically and chemically. The enzymatic method uses enzymes or bacteria for deproteinization by adding enzyme or by the involvement of chitinase to degrade chitin. Meanwhile, the chemical process is through demineralization by adding acid or alkali, such as HCl and NaOH [2].

A study by Arbia et al. [3], which isolate chitinolytic bacteria to produce several bacteria, one of them is *Pseudomonas aeruginosa* bacteria isolated from crab's cell. The production of chitinase enzyme from *Aspergillus niger* utilizing the blue swimmer crab's waste as inducer [4]. Protease produced by *Pseudomonas aeruginosa* K-187, the highest protease activity was as high as 21.2 U/mL, 10-fold that (2.2 U/mL) obtained before optimization [5]. In common with all enzymes, external factors such as temperature, pH and type of media are important for the activity, catalytic efficiency, stability and proper functioning of proteases [6]. Chitinase activity of isolates chitinolytic bacteria can degrade different chitins.

The needs for the chitin derivatives are increasing. Thus, researches on chitinase enzyme activity through the isolation of bacteria from the blue swimmer crab is needed. Observe primarily that two isolates chitinolytic bacteria from the skin of blue swimmer crab in Katialada village of Kwandang sub-district of North Gorontalo Regency, Gorontalo Province of Indonesia. The results obtained from the purification process following 48 h incubation in the temperature of 30°C shows a clear zone that formed in the colony of the bacteria [7]. However, specific zones are only found in white and light brown-colored bacteria.

Furthermore, these two isolates are macroscopically and microscopically identified. The R1 isolate shows a white-colored colony; meanwhile, the R2 isolate shows a light brown-colored colony. Meanwhile, from the shape/form and elevation of the colony, there are no differences between isolates R1 and R2 both colonies have a circular shape and convex elevation. The result of gram staining in these two chitinolytic bacteria isolates R1 and R2 shows the gram-negative result. This is signified by changes of color of these two isolates into the red after the gram staining. The study shows that CI 11 of the R1 isolate has the largest Chitinolytic Index by 1, the chitinolytic index shows the ability of

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Biographies: Sudin, Junior Scientist, research interest: marine biotechnology, Email: sudynsultra@gmail.com; **Rita Marsuci Harmain**, Research Assistant, research interest: marine microbiology, Email: rmarsuci@yahoo.com.

***Corresponding author: Rieny Sulistijowati**, Associate Professor, research interest: biotechnology process. Universitas Negeri Gorontalo, Faculty of Fishery and Marine Science, Department of Fishery Product Technology, Indonesia, Central City of Gorontalo, Sudirman Street No. 06, Postal Code 96128. Tel: +62-435-821125, Email: rienysulistijowati@ung.ac.id.

the microbes to degrade chitin. The more enzyme produced, the wider the clear zone produced as more chitin is degraded.

This is due to the type of bacteria growth pattern and enzyme activity needs to be known to have a good degrading ability. Therefore, this study is aimed at tests the produced chitinase activity and identification chitinase producer bacteria of blue swimmer crab.

2 Methods and material

2.1 Station and laboratory

Isolate R1 was obtained from fresh blue swimmer crab's cells which come from the crabs' farmer in Katialada village of Kwandang sub-district of North Gorontalo Regency, Gorontalo Province of Indonesia. The identification biochemical test of the chitinolytic bacteria were carried out at the Fish Quarantine Station Laboratory Quality Control and Fisheries Product Security Class I Gorontalo Province of Indonesia, and the chitinolytic bacteria enzyme activity test are carried out at the Pharmaceutical Laboratory of Universitas Negeri Gorontalo.

2.2 Materials and methods

The tools used in this study were test tube, inoculum needle, vortex, measuring cup, beaker glass, Erlenmeyer, centrifuge, centrifuge tube, shaker, pH meter, micropipette, micro tip, stirrer, magnetic stabilizer, petri dish, oven, crooked spoon, Bunsen lamp, incubator, laminar air, analytical scales, UV-vis spectrophotometer, and microscope.

Materials used in this study were R1 isolated of blue swimmer crab's cell, chitin, chitin colloidal (chitin, condensed HCl, NaOH, distillate water), chitin agar (chitin colloidal, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, yeast extract, agar, distillate water), Luria broth (yeast extract, tryptone water, NaCl, distillate water), nutrient agar, aluminum foil, alcohol, crystal violet, iodine solution, glucose, sucrose, lactose, maltose, mannitol, triple sugar iron agar, motility indole ornithine, oxidative/fermentative, methyl red-Voges Proskauer, methyl-red, and sterile liquid paraffin.

2.3 Quantitative activity of chitinase enzyme test

One dose of inoculum is added into 100 mL of production medium (similar composition to a solid medium, but without agar) then incubated in the temperature of 30°C and centrifuged in the speed of 170 r/min. Every three hours, 2 mL of cell culture is sampled for 33 h. Then centrifuged in the temperature of 4°C using 10.000 r/min speed for 10 min, the formed supernatant is the raw extract of chitinase enzyme. The absorbance is then measured using spectrophotometer UV/Vis in wavelength of 660 nm [8], the sample is carried out twice repetitions.

2.4 Biochemical test

The biochemical test is carried out to identify and classify bacteria into their group of taxonomy. The principle of this biochemical test is if the bacteria are cultured in several media, the bacteria show macroscopic differences in their growth [9,10]. Carbohydrate fermentation test to find out the bacteria's ability in fermenting carbohydrate by preparing the carbohydrate broth which consists of glucose, sucrose, maltose, and mannitol; MR-VP (methyl red-Voges Proskauer) test to inoculate bacteria into a medium, which incubated in the temperature of 30°C for 24 h by adding methyl red reagent and KOH, to observe the bacteria ability in producing the mix acid and acetylenes; Citrate test by inoculating bacteria into a medium and incubated in the temperature of 30°C for 24 h by adding bromothymol blue (BTB) reagent, then observe the ability of the bacteria to use citrate as the only source of carbon; H_2S test to inoculate the bacteria into sulfide indole motility (SIM) which incubated for 24-48 h in the temperature of 30°C, then observe the ability of the bacteria to produce H_2S which signify by the existence of black sediment; oxidation/fermentation (O/F) test by inoculating the bacteria into the O/F medium, which incubated for 24 h in the temperature of 30°C, then observe the ability of the bacteria to use carbohydrate through fermentation or oxidation; TSIA (triple sugar iron agar) test by inoculating bacteria into TSIA media, which incubated for 24-48 h in the temperature of 30°C, then observe the ability of the bacteria in fermenting glucose, lactose, and sucrose; Indole test by inoculating bacteria into indole media, which incubated for 24 h in the temperature of 30°C, then observe the ability of bacteria in degrading the tryptophan amino acid in the medium.

3 Results and discussion

3.1 Quantitative activity of the chitinolytic bacteria enzyme

The activity of the chitinolytic bacteria enzyme is essential to be known to find out the ability of the bacteria to produce the enzyme in 33 h with an interval of 3 h. As shown in Figure 1, there were several increasing and decreasing stages in chitinase activity. The first inclination happened during the incubation time of 0 to 12 h. The second increase of the enzyme activity showed that the substrate was starting to be hydrolyzed to produce the chitinase enzyme. Hence, bacteria could digest nutrition. Patil et al. [11] found that bacteria produce extracellular chitinase to take on nutrition. Following this inclination, there is the first declining phase on the 15th to the 18th hour of incubation. The decrease of this enzyme activity is due to other compounds (aside from N-Acetyl glucosamine) that triggers the decrease of enzyme production.

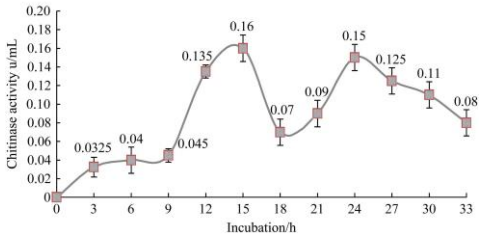

Figure 1 Chitinase activity curve from *Pseudomonas*

This phenomenon is due to the existence of other chitin-degrading enzymes produced by the bacteria. Colloidal chitin also can be hydrolyzed by deacetylating chitin produced by chitosan and chitosanase which produce chitobiose [12]. Following this declining phase, the chitinase activity climbs up in the incubation time of the 21st hour to its highest chitinase activity that can be obtained from the supernatant culture in the incubation of time of the 24th hour, which stated by the value of enzyme activity by 0.149 U/ml. One unit of chitinase enzyme activity is defined as several enzymes needed to release 1 mmol NAG/min. This result is different from the result of the enzymes activity test carried out by Purkan et al. [8] who found that the highest enzyme activity is in the 18th hour of incubation time, which started with the value of enzyme activity by 0.3850 U/mL moreover the ability of the bacteria to produce chitinase highly varied. Factors such as different types of bacteria, the growth rate of each isolate in the medium, temperature, pH or laboratory treatment during the experiment can be factors that influence variation in the produced enzyme activity [13,14]. Chitinase activity was 0.213 and 0.219 U/mL respectively of PBK 2 and SA 1.2 isolates from shrimp

waste. Based on 16S rDNA sequences, isolate of PBK 2 was identified as *Acinetobacter johnsonii* 3-1, whereas SA 1.2 was identified as *Bacillus amyloliquefaciens* GR53 with 99.78% similarity [15].

The rebound of enzyme activity shows that there is more of the substrate being hydrolyzed. The chitinase enzyme activity is steadily increasing until it reaches optimum incubation time. Following the reach of this optimum incubation time, the enzyme activity decreases due to the accumulation of hydrolyzed products, which can further inhibit the enzyme activity. This is characterized by the decrease of enzyme activity on the incubation time of hour 27 to hour 33. The decrease of chitinase enzyme activity after the optimum incubation time is due to the changes in the state of the enzyme ion and the state of substrate ion which caused denaturation of enzyme which followed by the loose of enzyme catalytic activity [12]. Besides, there are also change in the tertiary structure of the enzyme due to denaturation, which made the hydrophobic amino acid group within the enzyme to come into contact with water, thus, the solubility of the enzyme weakens. The decrease of chitinase solubility causes a gradual decrease in enzyme activity.

Chitinolytic bacteria isolates showed unstable chitinase activity (fluctuate). This may be due to the isolate that produces the chitinase at the beginning of its growth [13]. In line with the utilization of nutrition for growth, it is also suspected that chitinase is also used by bacteria as a source of protein, thus its chitinase activity decreases.

The decrease of enzyme activity can also be caused by factors such as temperature, pH, substrate and biomass during treatment in the laboratory. The temperature has two main influences on the reaction and the denaturation. The influence of reaction toward the enzyme is that the increase of temperature will accelerate the reaction process, while the decrease in the temperature will cause the reaction to slow down. When the temperature reaches a certain limit, it will cause denaturation. Besides, when the pH of the environment is too acid or base, enzyme denaturation can also happen. Reaction speed catalyzed by the enzyme is highly influenced by substrate concentration. In the low level of substrate concentration, reaction speed by catalyzed by the enzyme can also be very low. In reverse, reaction speed will increase along with the increase of substrate concentrate up to certain points that is the maximum reaction speed limit. When this saturated point of the enzyme has been reached, it will not function properly. Lastly, the number of bacteria inoculum (biomass) inserted into the media also strongly influence the enzyme activity.

3.2 Biochemical test

The biochemical test toward the characteristics of chitinolytic bacteria was carried out by fermenting bacteria in the various sources of nutrition as shown in Figure 2. The biochemical test result of chitinolytic bacteria is presented in Table 1.

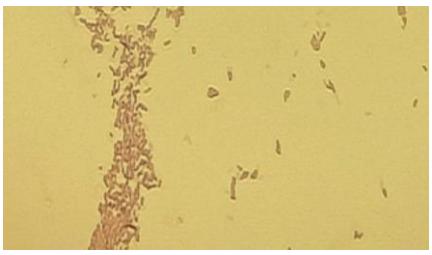

Figure 2 Isolate R1 (10×100 magnification)

Table 1 Biochemical characteristics of chitinolytic bacteria isolate R1

No	Test	Results
1	Glucose fermentation	Negative
2	Sucrose fermentation	Negative
3	Lactose fermentation	Negative
4	Maltose fermentation	Negative
5	Mannitol fermentation	Negative
6	Citrate use	Negative
7	Sulfide indole motility	Negative
8	Triple sugar iron agar	Alkaline/Alkaline
9	Methyl red reaction	Negative
10	Voges Proskauer reaction	Negative
11	Indole production	Negative
12	Oxidase/Fermentative action	Negative

Fermentation test in several types of carbohydrate (glucose, maltose, sucrose, mannitol, lactose) shows that all fermentation reaction is negative. This is characterized by the unchanging red color of the carbohydrate media. When the color of the medium in the carbohydrate test turns into yellow, it means that the colony forms acid from that carbohydrate [10].

A citrate test was carried out to find out the ability of the chitinolytic bacteria isolates to utilize citrate as the only source of carbon and energy. When a microorganism can use citrate, there will be an increase of pH and change in the color of the media into blue color. In this study, the citrate test reveals that the chitinolytic bacteria cannot utilize citrate as the only source of carbon. This is shown by the unchanging green color of the media, which means that the test result is negative. Positive test results in the citrate test are shown when the color changes from green to blue [16].

Further, the H_2S test result in SIM is negative. This negative result is reached when microorganism has no ability to hydrolyzed heavy metal within the media. H_2S is produced by several types of microorganisms, which can break or degrade amino acid within the sulfur (S). The existence of H_2S can be observed by adding several crystals of heavy metals into the media.

The reaction observable in the TSIA test shows a red color, which means that there is no change of color in both vertical and slight agar. This indicates that the bacteria are unable to ferment sugar. In the vertical agar if the bacteria can ferment glucose, the color of the media will change from red to yellow [17]. Whereas in slight agar, if the bacteria can ferment lactose and sucrose, the color of the media will change into yellow, meanwhile, when there is no fermentation process of lactose and sucrose, the color will not change.

Methyl red test also reveals a negative result. This is shown by the unchanging color of the media which does not change into yellow even after the addition of methyl red reagent. The red colour signifies the positive test result, and if the color of the broth is yellow, then the result of the test is negative [16]. Similarly, the Voges Proskauer test also shows a negative result. This is evident after the addition of the KOH solution; the color does not change. The Voges Proskauer test will be stated as positive when there is a form of acid, which signifies by the changes of medium color into pink after the KOH solution is added. Meanwhile, the indole test also shows a negative result. This result is obtained after the reagent Kovac is added, which signify by the formation of a yellow ring. The existence of indole is detected by Kovac reagent and the formation of a red ring.

The objective of the oxidizing fermentative test is to find out the oxidation and fermentation characteristics of bacteria toward glucose. Based on the result of the study on the O/F test, it does not show either oxidation or fermentation. This is evident when the media, either without liquid paraffin or without paraffin at all, do not change color. Bacteria are said to be fermentative when both inoculated media change color into yellow. Bacteria are oxidative when tube sealed with no paraffin changes color into yellow and the tube sealed with paraffin does not change color [18].

The morphology test result (macroscopic and microscopic tests) of chitinolytic bacteria have bar cell and gram-negative[9]. The biochemical test of the chitinolytic bacteria consists of carbohydrate, citrate, sulfide indole motility, triple sugar iron agar, MR-VP, indole and O/F test should obtain negative results for as indicators of a genus of Pseudomonas. Several studies to determine chitinolytic bacteria from the genus of Pseudomonas [4] that utilized blue swimmer crab's waste as an inducer to the production of chitinase enzyme from *Aspergillus niger*. A study by Arbia et al [3], which isolate chitinolytic bacteria to produce several bacteria, one of them is *Pseudomonas aeruginosa* bacteria isolated from crab's cell. Genus *Pseudomonas* generally has bar cell shape and gram-negative. A study by [19] which isolate *Pseudomonas aeruginosa* K-187 known produced two bifungtional chitinase and lysozyme. Thompson et al. [20] found that *Pseudomonas aeruginosa* strain 385 produced chitinase antigen.

4 Conclusions

This study concludes that chitinolytic bacteria isolated from blue swimmer crab's cell (*Portunus pelagicus*) genus of Pseudomonas. The highest chitinase activity was obtained from the supernatant culture obtained in the 24th hour, in which enzyme activity value is 0.149 U/mL as a good potential to degradable chitin ability.

Acknowledgements

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Chitinase activity of chitinolytic bacteria *Pseudomonas sp.* from the blue swimmer crab cell (*Portunus pelagicus*)

Rieny Sulistijowati[1]*, Sudin[1], Misri Gozan[2], Yuszda Salimi[3]

[1]Fish Processing Department, Universitas Negeri Gorontalo, Gorontalo 96128, Indonesia

[2]Chemical Engineering Department, Faculty of Engineering, Universitas Indonesia, Depok Campus, 16424, Indonesia

[3]Department of Chemistry, Universitas Negeri Gorontalo, Gorontalo 96128, Indonesia

* rinysulistijowati@gmail.com

ABSTRACT

This study is aimed at investigating the chitinase enzyme activity produced by chitinolytic bacteria from the skin of the blue swimmer crab (*Portunus pelagicus*) and identification of the genus isolate. This study consists of three stages; first, the screening of chitinolytic bacteria (isolation and macroscopic and microscopic identification); second, the qualitative and quantitative activity of the chitinase enzyme; third, biochemical identification of the bacteria. The quantitative chitinase enzyme activity is measured using spectrophotometer UV-Vis in the wavelength of 660 nm. The Chitinase enzyme is obtained from the isolation of chitinolytic bacteria, cultured within a media to grow solid chitin, which contains colloidal chitin substrate as a chitinase inductor at 30^{o} C. The chitinolytic bacteria isolate produces the highest chitinolytic index of 1. The highest chitinolytic activity is obtained from the 24 hour supernatant culture, with a value of enzyme activity at 0.149 U/mL. Macroscopic and Microscopic identification show that the chitinolytic bacteria isolated are negative gram bacteria in the form of stem, whereas the biochemical cell shows the characteristics of the Pseudomonas genus.

Keywords: Chitinolytic Bacteria; Blue Swimmer Crab's Skin; Chitinolytic Index; Chitinase Activity; *Pseudomonas* sp.

I. INTRODUCTION

Implementation of biotechnology toward chitin, which keeps progressing, is the utilisation of enzymes from a microorganism for biodegradation. In biodegradation, an enzyme derived from microorganism breaks a large molecule or chitin polymer into utilisable products. In general, the chitinase enzyme degraded the chitin(Purkan, *et al.*, 2016).

Microorganisms that degraded chitin, in general, are those derived from bacteria group. The Chitinase enzyme produced by chitinolytic bacteria has the potential to degrade chitin due to the existence of the chitinase enzyme, which enables the conversion of abundantly available chitin into usable products. The bacteria that produce chitinase enzyme or chitinolytic bacteria can be found within the habitat that contains a high level of chitin, such as in the cell of the blue swimmer crab. The blue swimmer crab cell (Portunus Pelagicus) can be obtained from the processing waste or fresh. Chitinase enzyme application can be informed of enzymatic production of chitin. The chitin can be produced enzymatically and chemically. The enzymatic method uses enzyme or bacteria for deproteinisation by adding enzyme or by the involvement of chitinase to degrade chitin. Meanwhile, the chemical process is through demineralisation by adding acid or strong acid, such as HCl and NaOH (Younes *et al.*, 2012).

Chemical synthesis o ; environmentally friendly method, as it uses]]] edients (HCl and NaOH) (Soeka & Triana, 2016). Thus, an enzymatic method is

considered better as it is easy, simple, fast and without any chemical solvent that negatively impacts the environment, thus, minimises the danger in relation to chemical utilisation. The downside of this method for industrial usage is that this method is quite expensive and needs an optimum condition for the enzyme to work maximally, and it is easy to be influenced by the environment (Chasanah, et al., 2014).

The needs for the chitin derivatives are increasing. Thus, research on chitinase enzyme activity through the isolation of bacteria from the blue swimmer crab is needed. Several studies have tried to hydrolyse chitin with the enzyme produced by *Aspergillus sp, Bacillus sp, Clostridium sp, Serratia sp*, *Aeromonas sp* and *Trichoderma sp*. Purkan, *et al.*, 2014).

Isolates of chitinolytic bacteria have the ability to degrade different chitins. This is due to the type of bacteria, growth pattern and enzyme activity. Enzyme activity needs to be known in order to have a good degrading ability. Therefore, this study aims to isolate chitinase producer bacteria and test the produced chitinase activity.

II. RESEARCH METHOD

Data obtained from this study are quantitative and qualitative data presented in the form of tables and figures.

Research Site

The isolation, identification, gram colouring and biochemical test of the chitinolytic bacteria are carried out at the Fish Quarantine Station, Quality Control and Fisheries Product Security Class I

Gorontalo; furthermore, the chitinolytic bacteria enzyme activity tests are carried out at the Pharmaceutical Laboratory of Universitas Negeri Gorontalo.

Tools and Ingredients

The tools used in this study are test tube, Ose needle, vortex (V-1plus), measuring cup, Beaker glass, Erlenmeyer, centrifuge (C2015), centrifuge tube, *shaker*, pH meter, pH meter, micropipette, micro tip, stirrer, magnetic stabiliser (Jisico), petri dish, oven, crooked spoon, Bunsen lamp, incubator (IB11E), Laminar Air Flow (LAF), analytical scales, UV VIS spectrophotometer (Lambda 45, Perkin Elmer) and microscope (Olympus Binocular CX22).

Ingredients used in this study are a blue swimmer crab's cell, chitin, chitin colloidal(chitin, condensed HCl *Merck* 100317 KgaA 64271 Darmstadt, NaOH *Merck* 106498 KgaA 64271 Darmstadt, aquades), chitin agar media (chitin colloidal, KH_2PO_4 *Merck* 104877 KgaA 64293 Darmstadt, $MgSO_4.7H_2O$ *Merck* 105886 KgaA 64271 Darmstadt, Merck yeast extract LP0021 Oxoid, agar Merck GRMO26 Himedia, aquades), Luria Broth (Yeast extract, tripton, NaCL, aquades), NA (Nutrient agar) Merck KgaA 64271 Darmstadt, aluminum foil, alcohol, crystal violet, iodine solution, safranin, Glucose Merck KgaA 64271 Darmstadt, Sucrose Merck KgaA 64271 Darmstadt, Lactose Merck KgaA 64271 Darmstadt, Maltose Merck KgaA 64271 Darmstadt, Mannol Merck KgaA 64271 Darmstadt, TSIA medium (Triple Sugar Iron Agar) Merck M021 Himedia, MIO medium (Motility Indole

Ornitin) Merck KgaA 64271 Darmstadt, O / F (Oxidative / Fermentative) medium Merck KgaA 64271 Darmstadt, MR-VP medium (Methyl Red-Voges Proskauer), methyl-red reagent Merck KgaA 64271 Darmstadt, Merck KgaA 64271 Darmstadt reagent and sterile liquid paraffin.

Preparation of Blue Swimmer Crab's Cell Sample (P.pelagicus)

Fresh blue swimmer crab cells are obtained from the crab farmer in Katialada village of Kwandang sub-district of Gorontalo Utara Regency. This sample was stored in a cool box with ice cubes layered with sealed and sterilised plastics. This is to prevent the sample from having direct contact with the ice blocks.

Preparation of Colloidal Chitin

Preparation of colloidal chitin uses a partial hydrolysis method. According to Arnold and Solomon (1986), colloidal chitin is produced from dissolving 10 grams of chitin into 200 mL of HCL, into 200 mL of concentrated HCl into a beaker glass; then the solution is settled for a night in a closed glass at 4 ^{0}C. This solution is filtered using *glass wool*, then the filtrate is mixed with 100 mL of cool aquades and added with NaOH 12 N to reach the pH 7. The solution is then centrifuged in the speed of 8.000 rpm for 20 minutes. The supernatant is then disposed of, after which aquades is added to the sediment = before it is then centrifuged again using at 8000 rpm for 20 minutes. The formed sediment is ready to use colloidal chitin.

Isolation of Chitinolytic Bacteria Produced from Blue Swimmer Crab Cell (Portunus pelagicus)

Inoculation is carried out on media that contains colloidal chitin in the dilution of 10^{-1} to 10^{-6}. The isolation process of the bacteria is carried out using selective, isolated media called chitin agar media. The process of chitin agar media creation is by dissolves 2.5 grams of colloidal chitin into 5 mL of aquades. Subsequently, 0.5 grams of $MgSO_4.7H_2O$; 0,1 g KH_2PO_4; 0.5 grams of yeast extract; 7.5 grams of agar and 495 mL of aquades are added. All the mixed ingredients are then boiled. The media is sterilised in the temperature of 121 ºC for 15-20 minutes. Five mL of sterilised media is then pour into each petri dish. The last three dilution concentrates (10^{-4}, 10^{-5}, 10^{-6}) are inoculated into chitin agar media by 1 mL using a micropipette, then each petri dish is wrapped and incubated in the temperature of 30 ^{0}C for 48 hours.

Screening of Chitinolytic Bacteria

The purification of chitinolytic bacteria uses the strike plate method. The result from the isolation of these bacteria then undergoes purification of culture bacteria to obtain pure colony isolate. The first step in the purification of bacteria is selecting different bacteria colonies from the isolation process. The bacteria colony is then inoculated in a surface of chitin agar medium using a sterilised Ose needle through the strike plate method to obtain a separated colony. The result from this process is then incubated in the temperature of 30 ^{0}C for 48 hours (Kamil *et al.*, 2007). The next processes are macroscopic and microscopic observations.

Macroscopic identification is directly carried out, whereas microscopic identification is carried out through gram colouring. The

gram colouring is carried out by taking one Ose of isolated bacteria, which is diluted into 3 mL of sterilised aquades, after which 10 µl are taken and put into a glass object and fixated. Subsequently, one drop of crystal violet (Gram A) is added into the solution for 1 minute then washed using flowing water and air dried. When it is dried, one gram of iodine (Gram B) is added, then washed with flowing water. Further, the bacteria isolate is added with 95% acetone alcohol solution (Gram C) for 30 seconds and washed with flowing water. After that, the isolate bacteria are added with Safranin (Gram D) for 2 minutes and washed using flowing water and air dried. The isolate is then observed under the microscope. Gram-positive bacteria will form a purple colour, whereas gram-negative bacteria will form a red colour (Cappuccino and Sherman, 2005)

Qualitative Activity of Chitinolytic Bacteria

A Chitinase activity test of chitinolytic bacteria is carried out by putting a drop in chitin agar media. This test is to see the clear zone produced. In this test, one dose of the pure isolate is taken and inserted into the petri dish, which contains solidified chitin agar media. The bacteria are incubated in the temperature of 30 ^{0}C for 72 hours. The established clear area is observed and measured using the ruler (Chasanah, 2009).

The clear zone formed in the medium is the response toward the colloidal chitin added into the medium. From all pure isolates tested of their chitinolytic activity, one isolate that produces the largest chitinolytic index is take

Quantitative Activity of Chitinase Enzyme Test

One dose of inoculum is added into 100 mL of production medium (similar composition to solid medium, but without agar) then incubated in the temperature of 30 ^{0}C and centrifuged in the speed of 170 rpm. Every three hours, 2 mL of cell culture is sampled. The sampling is started from hour 0 to hour 33. Then centrifuged in the temperature of 4 ^{o}C using 10.000 rpm speed for 10 minutes, the formed supernatant is the raw extract of chitinase enzyme. The absorbance is then measured using Spectrophotometer UV/Vis in λ 660 nm (Purkan et al., 2014).

Biochemical Test

The biochemical test is carried out to identify and classify bacteria into their group of taxonomy. The principle of this biochemical test is, if the bacteria are cultured in several media, the bacteria will show macroscopic differences in their growth (Cappuccino and Sherman, 2005; Adyta *et al.*,2017).

1. Carbohydrate Fermentation Test

The principle of this carbohydrate fermentation test is to find out the bacteria's ability in fermenting carbohydrate by preparing the carbohydrate broth that consists of glucose, sucrose, maltose and mannitol.

2. *Methyl Red*-Voges Proskauer (MR-VP) Test

The principle of this MR/VP test is to inoculate bacteria into a medium, which is incubated in the temperature of 30 ^{o}C for 24 hours by adding methyl red reagent and KOH, to observe the bacteria's ability to produce the mix acid and acetylenes.

3. Citrate Test

The principle of this citrate test is to inoculate bacteria into a medium and incubate in the temperature of 30 ºC for 24 hours by adding *Bromothymol blue (BTB) reagent,* then observe the ability of the bacteria to use citrate as the only source of carbon.

4. Hydrogen Sulfide (H_2S) Test

The principle of this H_2S test is to inoculate the bacteria into SIM (Sulfide Indole Motility), which is incubated for 24-48 hours in the temperature of 30 ºC, after which the bacteria's ability is observed to produce H_2S, which is signified by the existence of black sediment.

5. Oxidation/Fermentation (O/F) Test

This O/F test is carried out by inoculating the bacteria into O/F medium, which is incubated for 24 hours in the temperature of 30 ºC, then observe the ability of the bacteria to use carbohydrate through fermentation or oxidation.

6. *Triple Sugar Iron Agar* (TSIA) Test

This TSIA test is inoculating bacteria into TSIA media, which is incubated for 24-48 hours in the temperature of 30ºC, then the bacteria's ability observed in fermenting glucose, lactose and sucrose.

7. Indole Test

The principle of this indole test is to inoculate bacteria into indole media, which is incubated for 24 hours in the temperature of 30 ºC, then

the bacteria's ability observed in degrading the tryptophan amino acid in the medium.

III. FINDINGS AND DISCUSSION

Isolation Result and Purification of Chitinolytic Bacteria

The isolation result following 48 hours of incubation at 30 ^{0}C produces bacteria that grow in the chitin agar media. It is found that bacteria only grow in a petri dish 10^{-4}. These bacteria are then separated based on the appearance of their colony, shape and colour. The result is classified into three different colonies, namely white, cream and yellow.

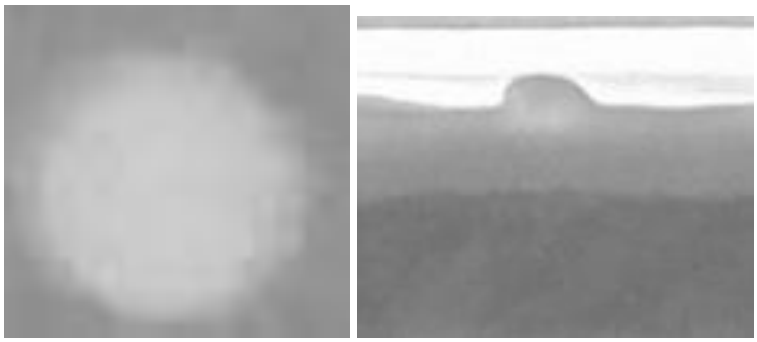
The result obtained from the purification process following 48 hours of incubation at 30 ^{0}C shows a clear zone that formed in the colony of the bacteria. However, clear zones are only found in white and cream coloured bacteria. Further, these two isolates are macroscopically and microscopically identified.

Bacteria colony

Macroscopic and Microscopic Identification Result

Based on the result of macroscopic and microscopic identification, it shows that chitinolytic bacteria shapes are round, white and cream coloured; the edge of the colony are complete and have gram negative. The morphology of the colony and the cell of chitinolytic bacteria are presented in Table 1.

(a) (b)

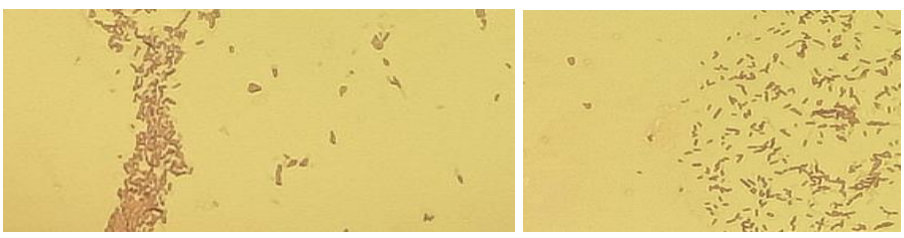


(1) (2)

Figure 1. Chitinolytic Bacteria Isolate. (a) Isolate R1, (b) Isolate R2, (1) Shape of the Colony, (2) Colony Elevation.

As seen in this table, the R1 isolate shows a white coloured colony, meanwhile, the R2 isolate shows a cream-coloured colony. Meanwhile, from the shape/form and elevation of the colony, there are no differences between isolate R1 and R;, both colonies have a circular shape and convex elevation.

The staining/colouring result of the bacteria is shown in Figure 2.



(a) (b)

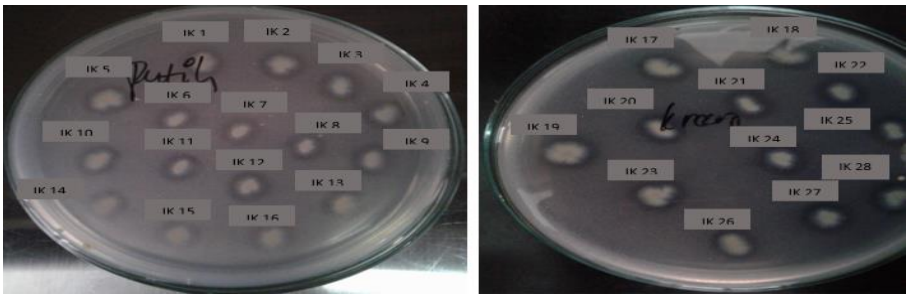
Figure 2. Gram staining/colouring of the Chitinolytic Bacteria, (a) Gram Negative of the isolate R1, (b) Gram-negative of the isolate R2. (10 x 100 magnification).

The result of gram staining in these two chitinolytic bacteria isolates R1 and R2, shows the gram-negative result. This is signified by colour changes of these two isolates into red after the gram staining.

Cappuccino and Sherman (2005) argue that gram-negative changes its colour into red due to the violet crystal colour that is dissolved during the administration of acetone solution, and takes the red colour of the Safranine. In general, the gram-negative bacteria have a high lipid cell wall. Hence, the lipid is dissolved in the acetone solution.

Qualitative Screening of Chitinolytic Bacteria Activity

Enzyme activity of chitinolytic bacteria is shown by the appearance of a clear zone formed around the colony. This clear zone proves that the isolate is able to degrade the chitin substrate within the chitin agar media. Harman *et al*. (1993) argue that chitinolytic bacteria are competent bacteria to produce chitinase enzyme and utilise chitinase to assimilate chitin as a source of carbon and nitrogen. The enzyme activity of the chitinolytic bacteria is seen in the white and cream-colour of the isolates as seen in Figure 3.



(a) (b)

Figure 3. The clear zone formed in chitin agar media by chitinolytic bacteria, which shows different Chitinolytic Index (a) Isolate R1 (CI 1-16) (b) Isolate R2 (IK 17-28).

Bacteria isolates produced from the blue swimmer crab's cell show the existence of a clear zone. This clear zone is formed due to the chitinase enzyme activity released from the cells of the bacteria to degrade macromolecules of the chitin into smaller chitin molecules. Thus, bacteria are able to digest nutrition from these small molecules.

Isolation and selection result of the bacteria cultured in chitin media points out that there are bacteria that grow with a different chitinolytic index (Table 2). This is due to the different level of bacteria adaptation toward their environment. In a selective solid media, it is known that bacteria are potential as chitinase producer, which signify by the formation of a clear zone around the colony. The more enzymes produced, the wider the clear zone that will be formed, as the number of degraded chitin is increasing (Harman *et al*. (1993).

The study shows that CI 11 of the R1 isolate has the largest Chitinolytic Index by 1. Chasanah (2009) who found that the largest chitinolytic index produced from the bacteria is 2.58, supports this result. The difference in this chitinolytic index is the different level of bacteria adaptation toward its environment. In addition, this difference is also due to the different types of bacteria. The chitinolytic index shows the ability of the microbes to degrade chitin. The more enzymes produced, the wider the clear zone produced as more chitin are degraded.

Table 1. Chitinolytic Index of the Bacteria

Note: Isolate R1 (Chitinolytic Index/CI 1-16), Isolate R2 (Chitinolytic Index/CI17-18).

The activity of the chitinolytic bacteria enzyme is presented in Figure 4 below.

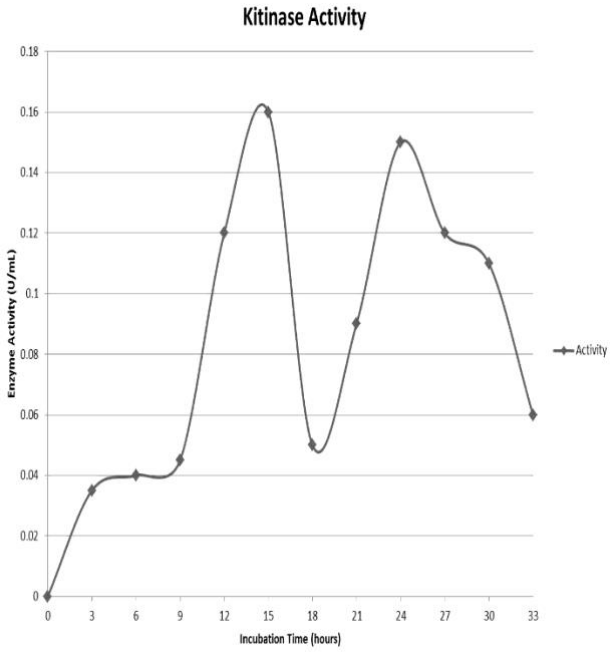


Figure 4. Chitinase Enzyme Activity Curve

The activity of the chitinolytic bacteria enzyme is essential to be known in order to find out the ability of the bacteria to produce the enzyme in 33 hours with an interval of 3 hours. There are several increasing and decreasing stages in chitinase activity. The first inclination happens on the incubation time of 0 hours to 12 hours. The second increase of the enzyme activity shows that the substrate is starting to be hydrolysed to produce chitinase enzyme. Hence, the bacteria are able to digest nutrition. Patil *et al*. (2000) wrote that bacteria produce extracellular chitinase to take on nutrition. Following this inclination, there is the first declining phase on the 15^{th} to the 18^{th} hour of incubation. The decrease of this enzyme activity is due to other

compounds (aside from N-Acetyl glucosamine) that trigger the decrease of enzyme production.

This phenomenon is due to the existence of other chitin-degrading enzymes produced by the bacteria. Fukamizo (2000) argues that colloidal chitin can also be hydrolysed by deacetylating chitin produced by chitosan and chitosanase, which produces chitobiose. Following this declining phase, the chitinase activity climbs up in the incubation time of a 21st hour to its highest chitinase activity that can be obtained from the supernatant culture in the incubation of time of a 24th hour, which is stated by the value of enzyme activity by 0.149 U/mL. One unit of chitinase enzyme activity is defined as the number of enzymes needed to release one mmol NAG/minute. This result is different from the result of the enzyme activity test carried out by Purkan *et al.* (2014), who found that the highest enzyme activity is in the 18th hour of incubation time, which stated with the value of enzyme activity at 0.3850 U/mL. In addition, Orinda, *et al.* (2015) argue that the ability of the bacteria to produce chitinase is highly varied. Factors such as different types of bacteria, the growth rate of each isolate in the medium or laboratory treatment during the experiment can be factors that influence variation in the produced enzyme activity.

The rebound of enzyme activity shows that there are more of the substrates being hydrolysed. The chitinase enzyme activity steadily increases until it reaches optimum incubation time. Following the reach of this optimum incubation time, the enzyme activity decreases due to the accumulation of hydrolysed products, which can further inhibit the

enzyme activity. This is characterised by the decrease of enzyme activity on the incubation time of hour 27 to hour 33. Fukamizo (2000) argues that this decrease of chitinase enzyme activity after the optimum incubation time is due to the changes in the state of the enzyme ion and the state of substrate ion, which caused denaturation of the enzyme, followed by the loose enzyme catalytic activity. In addition, there is also a change in the tertiary structure of the enzyme due to denaturation, which made the hydrophobic amino acid group within the enzyme come into contact with water, thus, weakening the solubility of the enzyme. The decrease of chitinase solubility causes a gradual decrease in enzyme activity.

Chitinolytic bacteria isolates show unstable chitinase activity (fluctuate). Orinda *et al.,* (2015) suggest that this may be due to the isolate that produces the chitinase at the beginning of its growth. In line with the utilisation of nutrition for growth, it is also suspected that chitinase is also used by bacteria as a source of protein, thus its chitinase activity decreases.

The decrease of enzyme activity can also be caused by factors such as temperature, pH, substrate and biomass during treatment in the laboratory. Temperature has two main influences on the reaction and the denaturation. The influence of reaction toward the enzyme is that the increase of temperature will accelerate the reaction process, while the decrease in the temperature will cause the reaction to slow down. When the temperature reaches a certain limit, it will cause denaturation. In addition, when the pH of the environment is too acidic or basic, enzyme

denaturation can also happen. Reaction speed catalysed by the enzyme is highly influenced by substrate concentration. In the low level of substrate concentration, reaction speed catalysed by the enzyme can also be very low. In reverse, reaction speed will increase, along with the increase of substrate concentrate up to certain points that is the maximum reaction speed limit. When this saturated point of the enzyme has been reached, it will not function properly. Lastly, the number of bacteria inoculum (biomass) inserted into the media strongly influences the enzyme activity.

Biochemical Test

Biochemical test toward the characteristics of chitinolytic bacteria is carried out by fermenting bacteria in the various sources of nutrition. The biochemical test result of chitinolytic bacteria is presented in Table 2.

A fermentation test in several types of carbohydrate (glucose, maltose, sucrose, mannitol, lactose) shows that all fermentation reaction is negative. This is characterised by the unchanging red colour of the carbohydrate media. Aditi *et al.*, (2017) argue that when the colour of the medium in the carbohydrate test turns into yellow, it means that the colony forms acid from that carbohydrate.

Citrate test is carried out to find out the ability of the chitinolytic bacteria isolate

Chitinolytic bacteria from the fermented liquid of organic waste. There are four isolates produced and the identification shows *Pseudomonas pseudomallei* bacteria. A study by Arbia *et al.* (2013), isolates chitinolytic bacteria to produce several bacteria, one of them being *Pseudomonas aeruginosa* bacteriam isolated from the crab's cell. Pseudomonas genus generally has bar cell shape and negative gram.

CONCLUSION

This study concludes that chitinolytic bacteria isolated from a blue swimmer crab cell (Portunus Pelagicus) have the ability to degrade chitin. This is proven by the formation of the clear zone with a chitinolytic index of 1. The characteristics of chitinolytic bacteria from this blue swimmer crab show that the bacteria are a bar shape negative gram bacteria from the Pseudomonas genus. The highest chitinase activity is obtained from the supernatant culture obtained in the 24th hour, in which enzyme activity value is 0.149 U/mL.

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Table 1. Quantitative Activity of the Chitinolytic Bacteria Enzyme

2)

Isolate	Type	Diameter Zone (mm)	Diameter colony (mm)	Index of chitinolytic
	IK 1	2	5	0,4
	IK 2	2	6	0,3
	IK 3	2	5	0,4
	IK 4	1,5	5	0,3
	IK 5	2	6,5	0,33
	IK 6	2	3	0,6
	IK 7	2	3,5	0,56
	IK 8	2,5	3	0,83
R1	IK 9	2	3,5	0,56
	IK 10	2	5	0,4
	IK 11	2,5	2,5	1
	IK 12	2,5	3,5	0,71
	IK 13	2	5	0,4
	IK 14	2	4	0,5
	IK 15	2,5	4,5	0,56
	IK 16	2	5	0,4
	IK 17	1,5	5,5	0,27
	IK 18	2	5	0,4
	IK 19	1,5	7	0,21
	IK 20	1,5	5,5	0,27
	IK 21	2	2,5	0,8
	IK 22	2	8	0,25
R2	IK 23	2	5,5	0,36
	IK 24	1,5	5	0,3

Table 2. Biochemical Characteristics of Chitinolytic Bacteria Isolate R1

No	Test	Results
1	Glucose fermentation	Negative
2	Sucrose	Negative
3	Lactose	Negative
4	Lactose fermentation	Negative
5	Maltose fermentation	Negative
6	Mannitol fermentation	Negative
7	Citrate Use	Negative
8	Sulfide Indole Motility	Negative
9	Triple Sugar Iron Agar	Alkaline/Alkaline
10	Methyl Red Reaction	Negative
12	Voges Proskauer Reaction	Negative
13	Indole production	Negative
14	Oxidase/Fermentative activity	Negative

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